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ATTACHMENT D

The Effect of *gacS* Inactivation on Biofilm Formation by *Pseudomonas aeruginosa*

ABSTRACT

Biofilms are the predominant form of microbial life and exhibit distinct physiology and phenotypes compared to the same micoorganisms growing as planktonic populations. The GacS/GacA two component regulatory system has been implicated in the formation of biofilms. The gene *gacS* encodes the sensor kinase component of this system. A *gacS*⁻ strain of *Pseudomonas aeruginosa* PA14 was generated and used to study the role of *gacS* in this phenomenon. The study of this mutant generated data that indicated gacS inactivation may reduce the ability of *P. aeruginosa* PA14 to form mature biofilms. However, *gacS* inactivation also leads to the accumulation of phenotypically stable phase variant bacteria that produce biofilms of greater mass than unaltered *P. aeruginosa* PA14. The results of this study indicate that *gacS* functions in the reversion of phase variant *P. aeruginosa* PA14 back to their 'normal' state.

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ABBREVIATIONS

ANOVA	analysis of variance
amp	ampicillin
BLAST	basic local alignment tool
bp	base pair
CA MHB II	cation adjusted Mueller Hinton Broth Two
CBD	Calgary biofilm device
CFU	colony forming units
CIAP	calf intestinal alkaline phosphatase
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EPS	extracellular polymeric substances
gm^R	gentamicin resistance cassette
GS-N	*gacS*⁻ phase variant strain of PA14 producing "normal" morphology colonies
GS-SV	*gacS*⁻ strain of PA14 producing "small" morphology colonies
IPTG	isopropyl-β-D-galactopyranoside
kb	kilobase
LB	Luria-Bertani broth/agar
mL	millilitre
NA	nutrient agar
OD	optical density

PA14	*Pseudomonas aeruginosa* PA14
PIA	pseudomonas isolation agar
PCR	polymerase chain reaction
RPM	revolutions per minute
SDS	sodium dodecylsulfate
Tris	(hydroxymethyl) aminomethane
TSB	tryptic soy broth
u	unit
ug	microgram
uL	microlitre
VBMM	Vogel Bonner minimal media
VBNC	viable but nonculturable
Xgal	5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside

1. INTRODUCTION

1.1 Impact of biofilms on medicine and industry

Microbial biofilms are collections of bacteria or fungi that exist in a multicellular or community form in an exopolysaccharide extracellular matrix, adherent to each other or a surface such as a medical device or tissue (1). This form of microbial life contrasts with the planktonic form, which is comprised of microbes floating free in a fluid. Despite the fact that a biofilm and a planktonic population of microbes may consist of the same species of organism, the behaviour and physiology of the two forms are often radically different (2, 3). This difference is at the core of biofilm research.

Biofilms are the predominant form of microbial life in nature (2). In medicine, biofilms are often implicated in chronic or implant associated infections (4, 5, 6, 7, and 8). Biofilm infections are resistant to chemical and biological removal compared to planktonic infections (2, 9). Parkins proposed that the antibiotic resistance of *P. aeruginosa* biofilms is not dependent on reduced penetration of antibiotic agents into the deeper regions of the film (31). Walters confirmed this observation by showing that the antibiotics ciprofloxacin and tobramycin penetrated throughout the depth of a *P. aeruginosa* biofilm without killing all the bacteria contained therein (71). Walters then speculates that the antibiotic resistance of biofilms is the result of low oxygen concentration and low metabolic activity in the deep portions of the biofilm. In any case, the mechanisms by which antibiotic resistance is mediated are still under investigation.

The impact of biofilms is not limited to medicine. Industry suffers from biofilm related problems as well. Biofilms have been shown to promote corrosion in fluid filled pipes (1,10,11), cause fuel flow obstruction in aircraft engines (Dr. M.E. Olson – personal communication), and have been determined to be a source of milking machine associated mastitis (12).

1.2 Physiology of biofilms

Biofilms are composed of individual bacterial cells suspended in matrix material composed of substances generated by the bacteria (63) and the environment (36). Strathmann found that the extracellular polymeric substances (EPS) of a *P. aeruginosa* biofilm are largely comprised of carbohydrates, proteins and uronic acids (63). Biofilms start to form when planktonic bacteria adhere to a surface, and begin replication to form a microcolony. These microcolonies enlarge and converge to form a contiguous biofilm.

The classic *P. aeruginosa* biofilm is composed of tall mushroom shaped microcolonies separated by water channels (75). It is thought that the water channels perform a function roughly analagous to that of a circulatory system in higher organisms (2). The cells occupying the sections of the biofilm furthest from the surface are thought to function at low metabolic levels with low dissolved oxygen concentration (71).

Bacteria living in the biofilm mode of growth are known to have altered gene expression patterns compared to planktonic cells (76). All in all, bacteria in the biofilm mode behave much like a eukaryotic tissue might be expected to behave (2). This coordination of function is accomplished through intercellular communication. One form

of this communication is called quorum sensing. In this process, freely diffusible molecules (called autoinducers) travel from one cell to another (3,13). When the autoinducers arrive at the receiving cell, they can cause that cell to alter its physiological behaviour. The denser the bacterial population, the higher the concentration of autoinducers, and the more likely the population is to exhibit a co-ordinated phenotype like a biofilm. The role of quorum sensing in the formation of biofilms is controversial. One author has shown that quorum sensing is critical in biofilm formation (3), while others have demonstrated it to be irrelevant (41, 78).

1.3 Two-component regulatory systems

As the name suggests, two-component regulatory systems consist of two parts. The first is a sensor kinase. The sensor kinase is a trans-membrane protein that is capable of receiving certain chemical signals from the environment. When such a signal is received, a histidine residue in the cytosolic domain of the protein is autophosphorylated. The phosporyl group is then transferred to an aspartate residue on the cognate response regulator molecule. The response regulator is the second part of the regulatory system. This cytosolic protein, when phosphorylated by the sensor kinase, alters the expression of certain genes (17, 18). The mechanism by which the response regulator alters gene expression, and therefore phenotype, varies from one system to another. Currently there are 123 known or suspected genes involved in two-component regulatory systems in *P. aeruginosa* (www.pseudomonas.com).

1.4 GacS/GacA Two-Component Regulatory System.

This research project has focussed on a single two-component regulatory system, the GacS/GacA system. The existence of this system was originally detected through a series of random mutagenesis experiments in *Pseudomonas syringae*. A mutation in the *gacS* gene (global activator of antibiotic and cyanide production) rendered the mutant strain deficient in lesion formation on bean plant leaves (19). Later, the projected translation of *gacS* was shown to have many characteristics in common with the sensor kinase proteins of known two-component regulatory systems (20). The GacS/GacA system was demonstrated to be important in the expression of genes encoding certain virulence factors as well as genes important in secondary metabolism in some pseudomonads (21,22,23,24,25,26,27,28,41, 42, 44). Among the various virulence factors associated with the GacS/GacA system are: extracellular enzyme production, cyanide production, antibiotic production, biofilm formation, swarming ability, and toxin production. Recently, it has been shown that the GacS/GacA system influences overall virulence in multiple and diverse hosts (43). Homologues to *gacS* have been found in multiple pseudomonads as well as other species of bacteria (29,66). Mutations in either *gacS* or *gacA* are usually expected to have the same or at least similar phenotypes (56, 54, 57). Spontaneous mutations in the Gac system have been seen under natural and laboratory conditions (54,56). The mutations are of multiple types (frameshift, deletion, duplication), but all have the same effect – stopping the function of the Gac system (56). These mutations can promote survival of the bacterial population as a whole (57). In the industrial production of bio-control organisms, these mutations can lead to a decrease in the effectiveness of these microbes (56). Finally, mutation of *gacA* has been shown to lead to the production of

viable but nonculturable (VBNC) cells (58). A summary of the proposed mechanisms of the GacS/GacA system is presented in Figure 1.

Recent work done in the Storey lab has suggested that the formation of biofilms by *Pseudomonas aeruginosa* is strongly influenced by the GacS/GacA two-component regulatory system (30). This research project was developed to further investigate the role of this system in biofilm formation by *P. aeruginosa*. This was accomplished by generating a *gacS* knockout mutant and studying its phenotype in both planktonic and biofilm modes of growth.

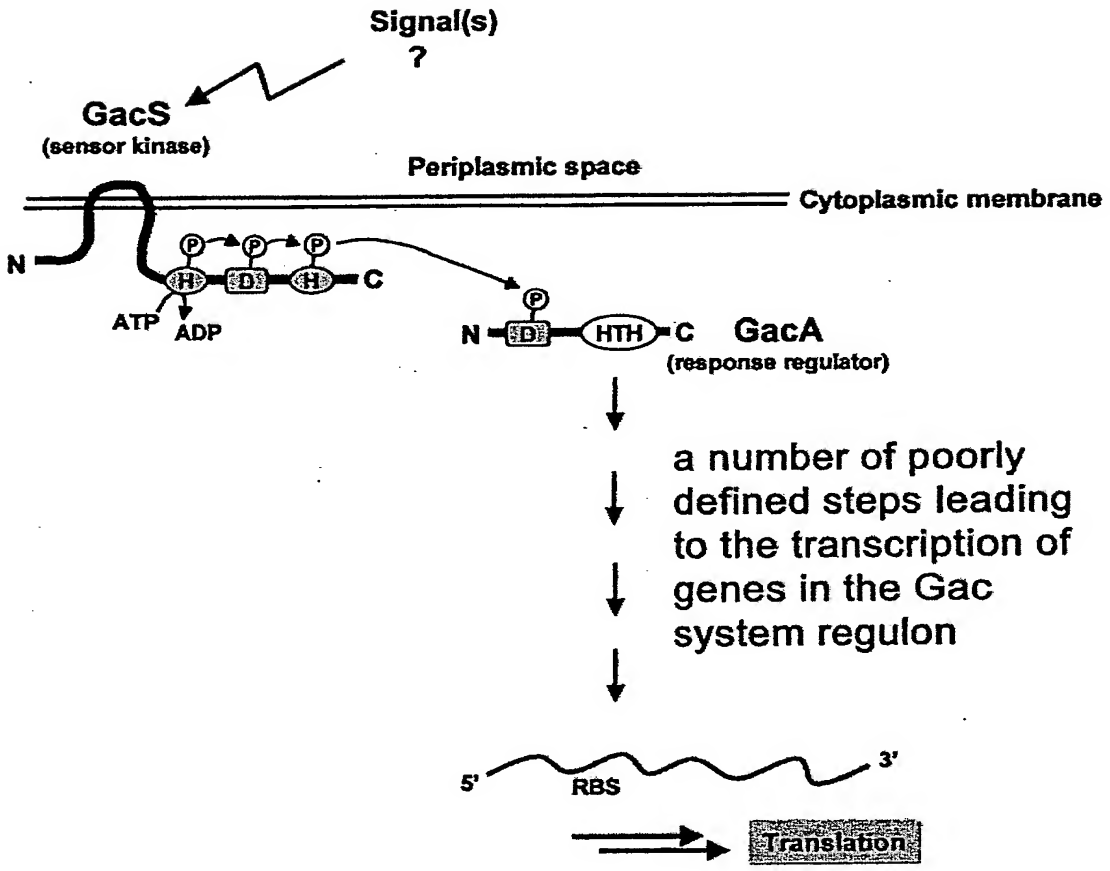


Figure 1. Schematic diagram of proposed mechanics of the GacS/GacA system. The complete mechanism by the GacS sensor can cause changes in gene transcription via the Gac A response regulator is not fully understood. Abbreviation used include: N = N-terminus, D = Aspartic acid, P = phosphate moiety, HTH = helix-turn-helix, C = C-terminus, H = Histidine, RBS = ribosome binding site. Modified from Haas et al. 2002 (77).

1.5 Phase variation

Phase variation is a process whereby phenotypic heterogeneity is generated within a microbial population. This is often a response to changing environmental conditions. Phase variation is often accomplished through reversible genomic rearrangements (67,70). Pseudomonas species phase variants have been seen in bacterial populations colonizing plant roots (54), and in chronic infections (53). The mutation of *pheN*, which has recently been re-classified as a homologue of the *gacS* gene (24), has been associated with phase variation in *P. tolaasii* (55). This study would seem to indicate that *gacS* in *P. aeruginosa* is also involved in this process. Specifically, it is proposed here that *gacS* is involved in the reversion of phase variants back to their 'normal' phenotype.

1.6 Overview of methods to be used in this study

The role of the *gacS* in the formation of *Pseudomonas aeruginosa* biofilms was studied by the functional inactivation of the *gacS* gene. This produced what is termed a *gacS* "knockout" (*gacS*$^-$) strain of *P. aeruginosa*. *P. aeruginosa* PA14 (a human isolate strain of *P. aeruginosa*) was used to make the knockout, as it has already been used to generate a *gacA*$^-$ strain (30). This knockout was compared over several parameters against the normal values for PA14 and a complemented strain. The *in vitro* ability of the *gacS*$^-$ strain to produce biofilms was studied, as were its planktonic and biofilm antibiotic sensitivity characteristics. The inactivation of *gacS* has previously been associated with altered antibiotic sensitivity (30). The impact of *gacA* in the production of biofilms has already been demonstrated by Parkins (30). However, the *in vivo* characteristics of this strain in mammalian biofilm infections have yet to be shown. An adaptation of a previously

published methodology for determining biofilm behaviour *in vivo* was used to determine these characteristics.

1.7 Hypothesis

Inactivation of *gacS* is *Pseudomonas aeruginosa* PA14 will cause a reduction in that organism's ability to form biofilms.

2. METHODS AND MATERIALS

2.1 Amplification of gacS from *Pseudomonas aeruginosa* PA14 genomic DNA

The first step in this study to examine the role of the *gacS* gene in the formation of biofilms by *Pseudomonas aeruginosa* was to create a null mutation through functional inactivation. This involved a number of molecular cloning steps that included disruption and inactivation of the *gacS* gene by insertion of a selectable marker, the gentamicin resistance cassette. This mutated *gacS* gene was then transferred into *E. coli* SM10 and, subsequently, by conjugation and homologous recombination, into *P. aeruginosa* PA14 to yield a *gacS*⁻ strain. A flow diagram of the steps involved in the whole procedure is shown in Figure 2. Methodology for the various individual steps is described in the sections that follow.

The project began with the amplification of *gacS* from *P. aeruginosa* PA14 genomic DNA. Although primers (MPGACS f/r) have already been described that will amplify a 3.4 kb segment of genome that contains the *gacS* gene in its entirety (30), new primers (prod 7f/r) were designed to amplify a smaller, more manageable segment of the *gacS* gene. This new fragment is predicted to be approximately 2.0 kb in size and to be completely within the termini of the *gacS* gene. The sequence of the primers used and the PCR cycle parameters employed in amplification of the gacS sequence are summarized in Table 1.

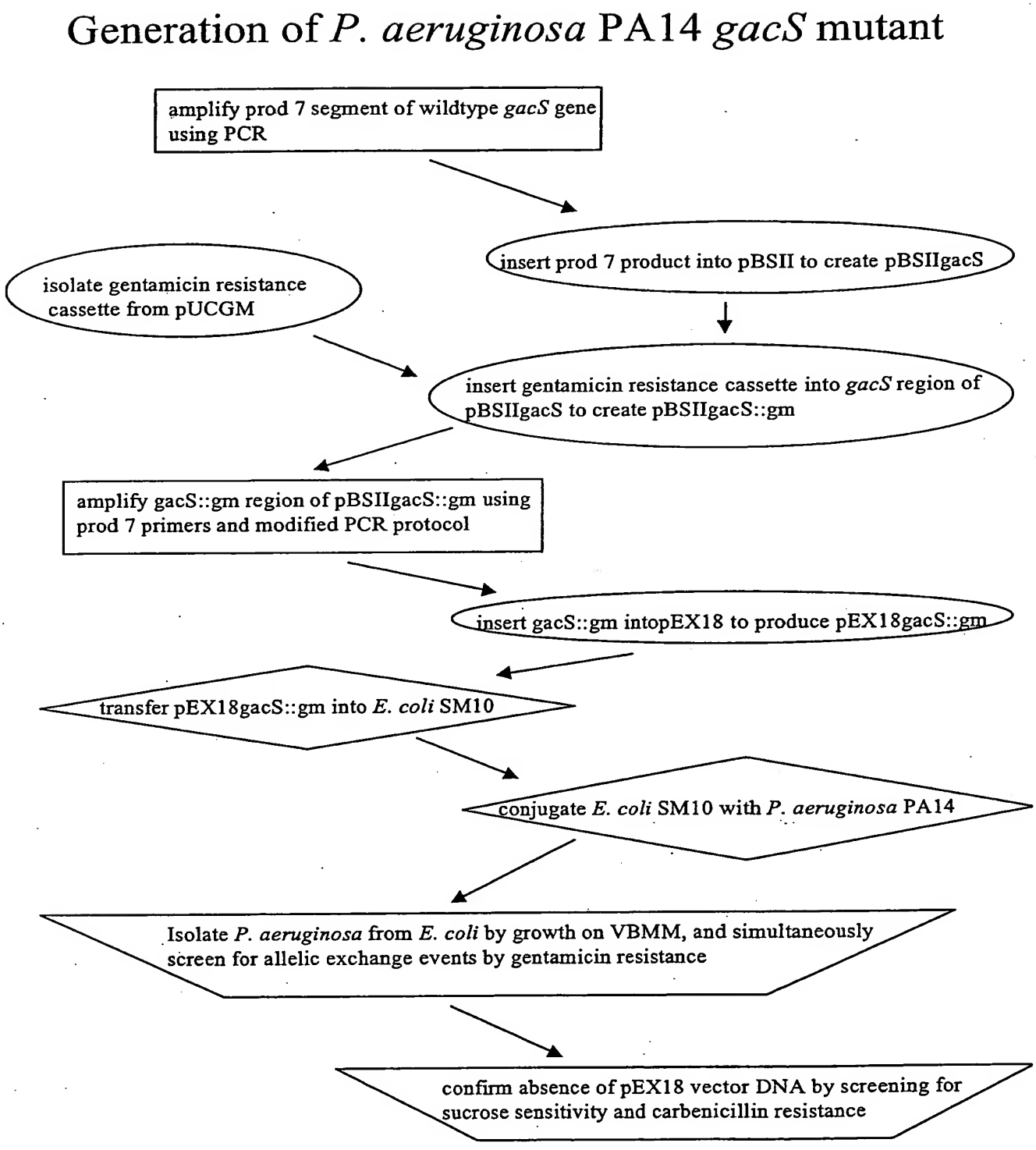


Figure 2. Diagram of steps in the generation of the *P. aeruginosa* PA14 *gacS* mutant.

Table 1. PCR primers and PCR cycle parameters used for *gacS* amplification.

Primers	Profile	Reference
prod 7f 5'-GATGGTGCTTGGCGGTTACTTCAC-3' Tm: 66.3°C **prod 7r** 5'-ACGTCCATGAAGACCAGGTCGAAG-3' Tm: 66.3°C	94°C for 5 min – 35x[94°C for 30 sec – 65°C for 30 sec – 72°C for 2 min] – 72°C for 7 min – 15°C hold	This study
MPGACS f 5'-CGCCAACCCCTCTTCCCCGTCTC-3' Tm: 71.7°C **MPGACS r** 5'-CGGCGACAGCGTGCGGCGAATAG-3' Tm: 71.7°C	94°C for 5 min – 30x [94°C for 30 sec – 63.5°C for 45 sec – 72°C for 3 min 45 sec] 72°C for 7 min – 15°C hold.	30

Reaction mixtures (50 uL total volume) were comprised of 5 uL 10x PCR reaction buffer containing 15 mM $MgCl_2$ (Qiagen), 1 uL forward primer (10 uM), 1 uL reverse primer (10 uM), 10 uL Q solution (Qiagen), 0.1 uL *Taq* DNA polymerase at 5 u/uL (Qiagen), and 26.9 uL water. When the inactivated, larger version of *gacS* was amplified it was necessary to increase the amount of *Taq* to 0.3 uL of 5 u/uL and increase extension time by 1 minute. The identity of the amplicon was confirmed by the comparison of sequence data to that for *gacS* previously published and available at www.pseudomonas.com.

Once the initial PCR product was verified, the same reaction was carried with Platinum Pfx polymerase (Invitrogen), a proof-reading DNA polymerase that does not leave thymine (T) overhangs on the 3' end of the product. The reaction conditions remained largely the same as above, with the minor modification of increasing the amount of Platinum Pfx polymerase content to 0.5 uL of 5 u/uL solution per reaction.

2.2 Construction of pBSIIgacS

Successful production of a "blunt ended" PCR product allowed the next step. The amplified *gacS* PCR product was inserted into the vector pBluescript II ks+ (Stratagene) by blunt-end ligation. A summary of the various vectors, E. coli host strains, and media used in this study are included in Tables 2, 3, and 4, respectively. Bluescript was cut with the restriction endonuclease Eco RV (New England Biolabs) and gel isolated. The reaction conditions for Eco RV restriction were as follows: a 50 uL reaction containing 5 uL NEBuffer #3, 0.5 uL of 10 mg/mL BSA, 2uL of Eco RV at 20,000 u/mL, and 43 uL of final miniprep solution (containing an unquantified amount of DNA) was incubated at 37^0C

Table 2. Vectors used in the generation of the *gacS* mutant

Name	Description	Reference
pBluescriptII ks+	cloning and sequencing vector in E. coli, expresses amp^R	Stratagene
pBSIIgacS	pBluescriptII ks+ containing a 2.0 kb portion of *gacS* amplified from PA14 genome; amp^R	This study
pBSIIgacS::gm^R	pBSIIgacS containing the gm^R cassette from pUCGM in the *gacS* region; gm^R	This study
pEX18	replicates easily in E. coli but suicides in P. aeruginosa. Used for allelic exchange mutagenesis. Constructed by ligation of 1791 bp PvuI fragment of pUC18 to large PvuI fragment of pEX100T; amp^R	34
pEX18gacS::gm	pEX18 containing the gacS::gm region from pBSIIgacS::gm; amp^R, gm^R	This study
pUCGM	plasmid containing Tn1696 derived gm^R gene flanked by pUC19 polylinker site amp^R gm^R	31
pUCP18	plasmid capable of replication in E. coli or P. aeruginosa. 1.8 kb stabilizing fragment from Pseudomonas incorporated into pUC18	46
pUCP18mpgacS	pUCP18 containing a 3.4 kb fragment amplified from *P. aeruginosa* PA14 containing the entire *gacS* gene and flanking sequences	This study

Table 3. *E. coli* and *P. aeruginosa* strains used in this study

Name	Genotype	Source or Reference
E. coli JM109	*endA*1 *recA*1 *gyrA*96 thi, hsd*R*17 ($r_k^- m_k^+$) *relA*1 *supE*44 Δ(*lac-proAB*) [F' *traD*36, *proAB*, *lacI*^{q}*Z* ΔM15]	49
E. coli XL1 blue	*endA*1 *recA*1 *gyrA*96 *thi*-1 hsd*R*17 *relA*1 *supE*44 lac [F' *proAB*, *lacI*^{q}*Z* ΔM15 Tn*10* (Tet^r)]	50
E. coli DH5α	φ80 Δ*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17 ($r_k^- m_k^+$) *supE*44 *thi*$^-$ *d*$^-$	51
E. coli SM10	*thi recA thr leu tonA lacY supE* RP4-2-Tc::Mu::*pir*	35
P. aeruginosa UCB-PP PA14	Human isolate able to elicit severe disease in plant and animal models	52
P. aeruginosa PA14 gacA-	PA14 ΔgacA::gm^R	52
P. aeruginosa PA14 GS-N	PA14 ΔgacS::gm^R, produces colonies the same size as those of unaltered PA14	This study
P. aeruginosa PA14 GS-SV	PA14 ΔgacS::gm^R, produces colonies smaller than those of unaltered PA14	This study

Table 4. Media used in this study*

Name	Constituents	Reference or Manufacturer
NA	8 grams Difco nutrient broth powder in 1 litre water	Difco
VBMM	MgSO4-7H2O (10 grams), citric acid-H2O (100 grams), K2HPO4 anhydrous (500 grams), NaNH4HPO4-4H2O (175 grams) 670 mL water.	47
LB	10 grams tryptone 5 grams yeast extract 10 grams NaCl	48
PIA	45 grams Difco pseudomonas isolation agar powder 20 mL glycerol 1 litre water	Difco
TSB	30 grams Becton Dickinson tryptic soy broth 1 litre water	Becton Dickinson
CA MHB II	22 grams Becton Dickinson cation adjusted mueller hinton broth II 1 litre water	Becton Dickinson

*Note: Unless otherwise stated, the agar version of these liquid media was generated by adding 15 g/L bacteriologic agar.

for 80 minutes followed by heat inactivation of the enzyme at 80^{0}C for 20 minutes. Gel isolation and purification was accomplished by using the Concert Rapid PCR Purification System (Invitrogen) or the QIAquick Gel Extraction Kit (Qiagen). The blunt PCR product was gel isolated and purified using the same kits. Both components were then used in a 20 uL ligation reaction containing: 1uL of T4 ligase (New England Biolabs, at 2,000,000 u/mL), 2uL of 10x ligation buffer (NEB), 12 uL of insert as collected from the gel extraction kit, and 5 uL of the linearized vector. The ligation was allowed to proceed at 15^{0}C overnight. A vector diagram of the expected recombinant plasmid, pBSIIgacS, is shown in Figure 3. The product of this reaction was used to transform competent *E. coli* XL1 Blue. Transformant colonies were recognized by blue/white screening on nutrient agar plates containing 100 ug/mL ampicillin. Plates containing ampicillin were treated with 40 uL of 40 mg/mL 5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside (X-gal) in dimethylformamide (DMF) and 4 uL of 200 mg/mL isopropyl-β-D-galactoyranoside (IPTG). Transformant colonies containing pBluescript II without insert were blue in colour through the action of β-galactosidase. Those containing pBluescript II that had an insert were white (through the inactivation of the gene for β-galactosidase coded for in the cloning site of pBluescript II). Candidate colonies (white) were screened based on plasmid size, and PCR product size following linearization of the plasmid with the restriction enzyme SphI (NEB). Digestion reactions were carried out in a volume of 50uL consisting of 5 uL NEBuffer #2, 2 uL of enzyme (5,000 u/mL), and 43 uL of plasmid DNA in water as isolated from the miniprep kit. Incubation was allowed to proceed for 80 minutes at 37^{0}C and the reaction stopped by heat inactivation at 65^{0}C for 20 minutes.

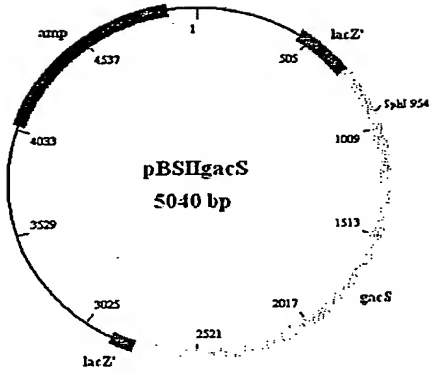
Figure 3. Vector diagrams of recombinant plasmids used in the generation of the *P. aeruginosa* PA14 *gacS* mutant.

a.) pBSIIgacS

b.) pBSIIgacS::gm

c.) pEX18gacS::gm

d.) pUCP18mpgacS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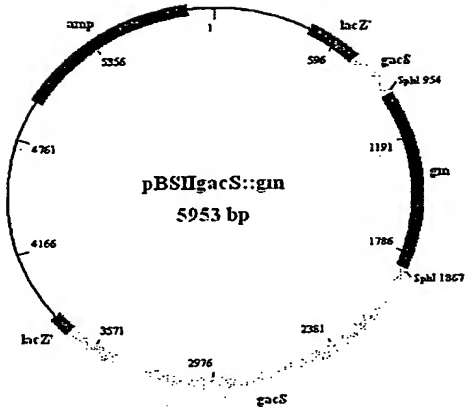
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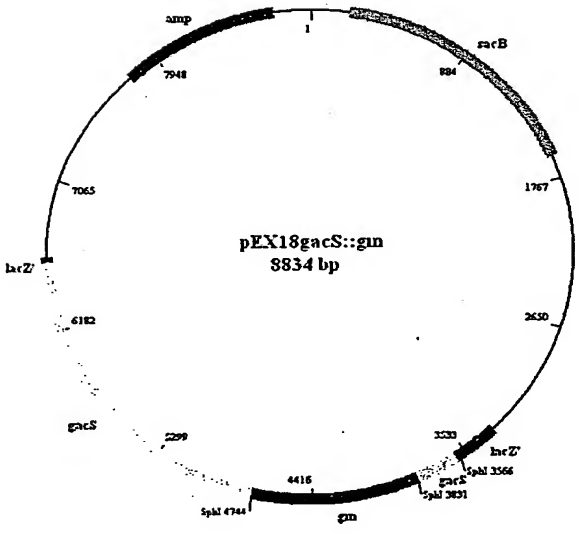
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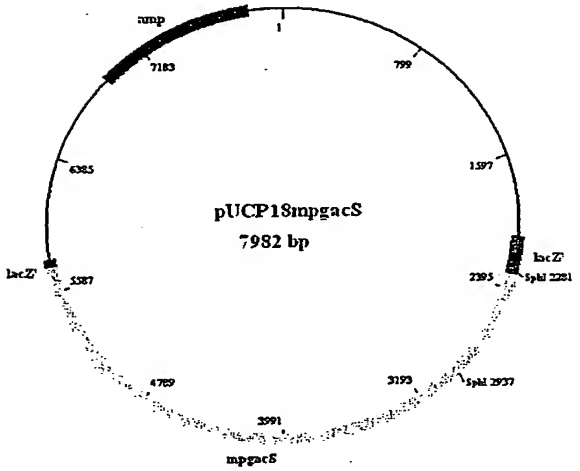
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3193
3991
4789
5587
6385
7183
SphI 2281
SphI 2937

d

2.3 Construction of pBSIIgacS::gm

After the construction of pBSIIgacS, the construction of pBSIIgacS::gm was initiated. The vector pUCGM was harvested from its host strain using the QIAprep Spin Miniprep Kit (Qiagen). The gentamicin resistance cassette (Gm^R) present in pUCGM (31) was separated from the rest of the vector by digestion with the restriction endonuclease SphI (New England Biolabs). Reaction conditions for this digest were the same as previously stated. The resulting free Gm^R cassette was gel isolated using the QIAquick Gel Extraction Kit (Qiagen). The construct pBSIIgacS was cut at a single point within the insert with SphI . Again, this linearized construct was gel isolated. The cassette and linearized pBSIIgacS were subjected to a "sticky-end" ligation reaction with T4 ligase, using reaction conditions that were identical to those used previously for T4 ligase blunt-end ligation. The resulting ligation products (see Figure 3b) were then used to transform *E. coli* JM109. Colonies were initially screened by growth on plates containing 15ug/mL gentamicin. Those colonies that grew on these plates were assumed to contain the Gm^R cassette and therefore the pBSIIgacS::gm construct. The direction and frame of insertion of this cassette does not matter as it contains its own promoter and has been shown to function in gram negative bacteria (32). Further screening on colonies was done by PCR, plasmid size, and digestion of plasmid.

2.4 Construction of pEX18gacS::gm

The final construct produced was pEX18gacS::gm. For this construct, the insert (gacS::gm) was produced by blunt PCR of the pBS-gacS::gm insert using the prod7f/r primers and Platinum Pfx DNA polymerase. The host vector, pEX18 (34) was cut with SmaI (New England Biolabs) in a 50 uL reaction that contained 43 uL of pEX18 miniprep DNA (of unknown concentration), 5 uL of NEBuffer #4, and 2 uL of SmaI at 10,000 u/mL. The reaction mixture was incubated at room temperature for two hours and the product gel purified. At this point, a standard ligation reaction using T4 ligase, the SmaI linearized vector and the blunt ended insert was carried out as previously stated. This vector has the characteristic of conferring sucrose sensitivity to the host, through the *sacB* gene (33,34). Bacteria containing this vector died when grown on LB plates containing 5% sucrose. After the ligation reaction, the product was used to once again transform *E. coli* JM 109. Transformants were initially screened by gentamicin resistance (LB agar containing 15ug/mL gentamicin), and sucrose sensitivity (5% in LB agar). Estimates of plasmid and insert size, PCR, and restriction digestion with Sph I, were again used to confirm this construct (Figure 3c). Once collected from the JM109 host, this construct was transformed into *E. coli* SM10 to allow for conjugation with *P. aeruginosa* (35) in the next step of this project. Competent *E. coli* SM10 were generated using the protocol outlined by Ellard (45).

2.5 Generation of *Pseudomonas aeruginosa* PA14 *gacS* mutant

The strain of *E. coli* SM10 carrying the pEX18gacS::gm construct was conjugated with *P. aeruginosa* PA14 using a procedure modified from Parkins (30). This protocol hinges on the fact that pEX18 constructs are suicide vectors in *P. aeruginosa*. That is, the plasmid itself cannot replicate in *P. aeruginosa*. Therefore, expression of the genes encoded on the plasmid can only occur if those genes have been transferred to the chromosome through a double crossover event (allelic exchange). Single crossover events, though more common than the doubles (35), are differentiated on the basis of sucrose sensitivity. Single crossover events will result in the expression of the *sacB* gene and a β-lactamase that will confer resistance to carbenicillin (500ug/mL) (34), whereas double crossovers will not. For this procedure, donor cultures of *E. coli* SM10 and recipient cultures of *P. aeruginosa* were grown overnight in rich media with and without 15 ug/mL gentamicin respectively. The *P. aeruginosa* strain used in this study is UCBPP PA14, a human isolate able to elicit sever disease in plant and animal models (52). Cultures were concentrated by centrifugation and deposited onto a TY plate to allow conjugation to occur on this substrate when incubated overnight at 37^0C. Bacteria on the plate were then scraped off the agar, resuspended in saline, and deposited onto VBMM agar plates containing 15 ug/mL gentamicin (which do not allow for the growth of *E. coli* and allows for *P. aeruginosa* mutants to grow slowly). Colonies that formed on these plates were then streaked on LB agar plates containing 5% sucrose. This allowed for the selection of allelic exchange events in the *gacS* region, given that the construct version of *gacS* contains a gentamicin resistance cassette within its insert. The double crossover event resulted in the replacement of the genomic *gacS* gene with this altered version. Thus colonies that formed on both VBMM agar containing gentamicin as

well as LB agar containing 5% sucrose consist of *Pseudomonas aeruginosa* that has undergone a double crossover event with pEX18*gacS*::gm. After gentamicin and sucrose sensitivity screening, further validation of potential knockouts was done via PCR, sequence analysis, and sensitivity to carbenicillin (500 ug/mL). The standard prod 7f/r primer set was used on genomic DNA collected from the suspected knockout. These primers produced a 3 kb product rather than the normal 2 kb product because of the incorporation of the 1 kb gm^R cassette. Sequence analysis verified the presence of the *gacS* sequence disrupted by the insertion of a gentamicin resistance cassette about 300 bases from one end of the product. At this point a single candidate clone (#12) was chosen for further evaluation. This clone was also resistant to carbenicillin (500 ug/mL).

2.6 Generation of complemented strains.

The plasmid pUCP18 (46) was isolated from *E. coli* DH5α using the QIAprep Spin Miniprep Kit (Qiagen). That vector was then cut with Sma I (NEB) and the ends dephosphorylated with Calf Alkaline Phosphatase (NEB). *gacS* and the surrounding sequences were amplified using Platinum Pfx polymerase (Invitrogen) and the primers MPGACS f and MPGACS r (30). This amplicon consisted of the *gacS* gene from PA14 plus approximately 320bp on each end of the gene. In this manner, the gene and the sequences directing its expression were amplified with high fidelity. The amplicon was then phosphorylated with T4 polynucleotide kinase (NEB). Ligation of the amplicon into the vector was accomplished using T4 ligase (NEB) in the manner previously described. This construct (Figure 3d) was transformed into *E. coli* DH5α and transformants selected by means of blue/white screening on LB media containing 100 ug/mL ampicillin, IPTG and Xgal. A single white colony was selected from the screening plates and grown in nutrient broth containing 100 ug/mL ampicillin. The construct was isolated using the QIAquick Gel Extraction Kit, and this used to transform competent *E. coli* SM10. The transformed *E. coli* SM10 was then conjugated with *P. aeruginosa* PA14, GS-N, and GS-SV (see page 57 for an explanation of this nomenclature). This was accomplished as previously stated, though selection of conjugants using VBMM agar containing 15 ug/mL gentamicin and 500 ug/mL carbenicillin. Conjugants were further confirmed by the isolation of the pUCP18mpgacS construct using the protocol outlined in the section, "Plasmid DNA isolation from *P. aeruginosa*". The genomic and plasmid DNA isolated by this method was used as template for an MPGACS PCR.

2.7 Evaluation of the *gacS* mutant

2.7.1 Biofilm generation using the Calgary biofilm device

The first phenotypic parameter to be evaluated was the rate of planktonic and biofilm growth of the *gacS* null mutant compared to the wildtype strain. The method of Parkins (30) was used. A 10 uL aliquot of overnight broth culture in TSB was used to innoculate 22.5 ml of TSB in the Calgary Biofilm Device (CBD) (79). The filled CBD was then incubated at 37^0C while being rocked on a Bellco Biotechnology Rocker Platform. The rocking motion produces the shear force necessary to produce biofilms on the CBD pegs.

2.7.2 Determination of CFU/mL and CFU/peg

At specified time points, pegs from the CBD were removed and sonicated to release the cells into sterile saline. Serial dilution was then used to enumerate the bacteria involved in the biofilm. Saline (180 uL) was instilled into each of the wells in a 96 well plate and 20 uL of planktonic culture or sonicated saline was added to the wells in the first column. Aliquots (20 uL) from the first column were transferred to the still sterile wells of the second column. This was repeated until a total of 9 columns were innoculated. A final 20 uL aliquot of the suspension from each well was then plated onto a nutrient agar plate (that may contain an appropriate antibiotic). The position of the droplet from each well was noted. The number of colonies formed from the least diluted well producing discrete colonies was noted. The column number roughly corresponds to the order of magnitude of CFU present (i.e. 5 colonies from the sixth column would indicate $5x10^6$ CFU/mL or peg). This number is multiplied by 5 to give a more accurate estimate of CFU per mL or peg as it would otherwise reflect only CFU/0.2 mL. Samples of liquid culture medium were

withdrawn from the CBD at the same time points and serially diluted in a similar manner to evaluate planktonic numbers. In an effort to minimize the effects of chance and biofilm variability associated with peg location in the CBD, a specific sampling procedure was adopted. Every effort was made to collect pegs from the same or similar locations on each CBD lid. Two separate pegs or broth culture samples were collected from each CBD. These pegs and samples were processed separately as stated above. Because the serial dilution procedure relies on thorough resuspension of bacterial cells, each dilution series was plated twice. The results of each replicate dilution series were averaged to represent the true CFU/peg or mL of the peg or culture aliquot being assayed. These results for each of the two samples collected were then averaged to provide a representative value for a given peg or culture aliquot at that time point for the culture in question.

To prove statistically significant differences in the planktonic and/or biofilm growth of the various strains tested, similar studies were undertaken. In these studies, only one or two time points were studied, but 8 or 12 pegs and broth culture samples were collected. Otherwise, the methods employed in these studies were the same as for the growth curve experiments. The methods for statistical analysis are detailed later in this chapter.

2.7.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC)

The next parameter to be measured was antibiotic sensitivity. Biofilms were grown as stated above until they were 17 or 28 hours old. At that point, both pegs and liquid culture medium from the CBD were collected. Pegs were placed into 96 well microtitre plates

containing serial dilutions of various antibiotics. Row position of each antibiotic was rotated in order to compensate for any differences in innoculum size due to position of the pegs on the plate. Liquid broth culture was placed in similar microtitre plates. Both the planktonic and biofilm populations were subjected to the same concentrations of the various antibiotics for the same time period. After incubation for 16-20 hours at 37^0C, both populations were assessed for growth. CBD lids were removed, sonicated in a 96 well biofilm recovery plate containing 200 uL of CA MHBII in each well, and the 96 well plate grown overnight with agitation. Optical density (OD) at 650nm of each well was determined using a Molecular Devices THERMOmax microplate reader. Liquid media was removed from the CBD plates, 20 uL innoculated into each of the wells of a 96 well plate containing the antibiotics, incubated , and assessed for growth in a manner identical to that of the biofilm recovery plates. The minimum inhibitory concentration (MIC) of a given antibiotic was defined as the lowest concentration of that antibiotic that did not allow for growth of planktonic organisms on the recovery microtitre plate. Similarly, the minimum biofilm eradication concentration (MBEC) was defined as the minimum concentration of a given antibiotic that did not allow for the growth of bacteria from material harvested from the device pegs (biofilms).. The drugs that were evaluated included: amikacin (Sigma) , aztreonam (ICN), ceftazidime (Eli Lilly), ciprofloxacin (Bayer), erythromycin (Sigma), gentamicin (Sigma), imipenem (Merck Frosst), pipercillin (Cyanamid Canada), polymixin B (Sigma), tetracycline (Sigma), and tobramycin (Sigma). Some of these antibiotics are commonly used to combat *P. aeruginosa* infections, others illustrate particular mechanisms of action. Drug concentrations were doubled in each consecutive well to produce a range of concentrations from 2 ug/mL to 1024 ug/mL (i.e. 2,

4, 8 ..... 1024). All antibiotics except erythromycin were dissolved in water and filtered through a 0.22um filter. Thirty per cent ethanol was needed to achieve dissolution of erythromycin.

2.8 Evaluation of *gacA* mutant biofilms in implant associated infections

The potential for biofilm development in the context of an implant associated infection was also explored. This was only done for the *gacA* null mutant used by Parkins (41). The protocol used by Ward (36) was modified to allow for less distress to the experimental animals, and to use rats instead of rabbits. Thirty-day old rats were anesthetized using halothane and an induction box. Once anesthesia was induced, the animal was maintained under halothane via facemask. A small patch on the animal's abdomen was clipped and scrubbed with betadine soap and rinsed with 70% ethanol. A 14 gauge hypodermic needle was placed percutaneously into the abdomen so that it penetrated the peritoneum but no internal structures. Two 10mm x 2mm x 2mm lengths of silastic tubing were placed into the bore of the needle using sterile forceps. A small amount (1mL) of fluid was used to inject the tubing sections into the abdominal cavity. The composition of the fluid was either a bacterial suspension in normal saline of known concentration (*gacA*$^-$, or unaltered) or sterile saline in the case of the control group. Each group consisted of 5 animals. The animals were then allowed to recover from anesthesia. Butorphanol was given at induction of anesthesia as prophylactic analgesia. Twenty-four hours later, the animals were euthanized by CO_2 asphyxiation, their abdominal cavities opened, and the implants removed. One implant from each animal was sonicated for 30 minutes in 1mL of sterile saline and used for enumeration of bacterial numbers. Bacterial numbers were

transformed into $\log_{10}$ values and analyzed by a one-way analysis of variance followed by testing for the least significant difference (see Appendix I). The other implant was fixed in glutaraldehyde for scanning electron microscopy.

2.9 Scanning Electron Microscopy

Samples were prepared for scanning electron microscopy by two different methods. Samples to be sputter coated were first fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer at 4^0C overnight. They were then allowed to air dry in a fume hood. The samples were then glued onto aluminum stubs with a mixture of epoxy resin (LePage 5 Minute Epoxy Glue) and silver paste (Colloidal Silver Paste – Electron Microscopy Sciences). Then, the samples were placed into a Technics Hummer I sputter coater and coated with gold/paladium. The coated samples were then placed in a scanning electron microscope (Philips XL 30 ESEM for pegs, Leo 360 for silastic tubing) and images obtained. Some peg samples were imaged in an "environmental mode" in which the electrons used for generating images were elicited as secondary electrons from water vapour introduced into the imaging chamber. In these samples, fixation and attachment to stubs was achieved in the same manner as for sputter coated samples except that no sputter coating was ever done. Specific settings for each of the images were recorded at the bottom of each photomicrograph.

2.10 Genomic DNA isolation

Genomic DNA was isolated using the protocol outlined in the instructions for Trizol reagent (Invitrogen). Briefly, bacteria were grown in broth culture and the cells pelleted by

centrifugation. Approximately 1 mL of Trizol reagent was then added to the pellet and the cells resuspended. The mixture was then allowed to sit for 5 minutes at room temperature, and 0.2 mL of chloroform added. The tubes were mixed and then spun in a centrifuge at roughly 10,000 rpm for 15 minutes. The phenol phase (not the interphase as this contained too much protein) was then extracted and mixed with 0.3 mL of 100% ethanol. The DNA was allowed to precipitate at -20^{0}C for a few minutes, then washed twice with a 1 mL solution of 0.1 M sodium citrate in 10% ethanol . The pellet was then washed in 70% ethanol, dried, and redissolved in 8mM NaOH.

2.11 Plasmid DNA isolation from *P. aeruginosa*

The kit used for most of the minipreps in this work was not suitable for transformed *Pseudomonas aeruginosa* strains. A protocol modified from the Cepko lab at the Harvard Medical School (http://axon.med.harvard.edu/~cepko/protocol/mike/D4.html) was, however, useful. Overnight cultures were pelleted by centrifugation and resuspended a solution of 0.1 N NaOH, 0.2% SDS, 10mM Tris, and 1 mM EDTA. The suspension was then treated with a solution of 3M sodium acetate pH 5.2. The resulting cloudy solution was processed through a phenol/chloroform/isoamyl alcohol extraction. 200 uL of the aqueous phase was treated with 600 uL of 100% ethanol. The DNA pellet formed was washed with 70% ethanol and allowed to dry. The dry pellet was then re-suspended in water. This procedure produced a sample that was mostly composed of plasmid DNA, but also contained a small amount of genomic DNA.

2.12 Assay for phage infection

To screen for the presence of a wild bacteriophage that may have infected the *gacS* mutant to produce colony variants, small variant and normal morphology *gacS* mutants were grown overnight at 37^{0}C in TSB. That culture was then plated onto nutrient agar containing no antibiotics. This plate was allowed to incubate overnight at 37^{0}C. The plate was then allowed to incubate at room temperature for 72 hrs. At the end of this time period, the plate was observed for zones of lysis. No zone of lysis was ever observed.

2.13 Exoprotease assay

Milk agar plates were made by mixing standard agar with milk and autoclaving. Nutrient agar solution was made at twice the normal concentration. Skim milk powder (Difco) was dissolved in water at 10% m/v concentration. Both solutions were autoclaved and then mixed in equal volumes. Bacteria were allowed to incubate overnight at 37^{0}C and then 10 uL spotted onto the plates at dilutions of 10^1- 10^4. Two spots of each strain were placed on each plate (one plate per dilution). Zones of clearing were measured with a ruler.

2.14 Transformation procedures

Competent *E. coli* strains were stored in a -70^{0}C freezer in individual use aliquots. One or more aliquot was allowed to thaw on water ice for 20-30 minutes. Plasmid DNA to be transformed into the host strain was added to the thawed cells and allowed to incubate on ice for 20-30 minutes. The mixture was then heat shocked at 42^{0}C for 45 seconds and then returned to ice for 1 minute. If selection of transformants was to be based upon resistance to ampicillin, the cells were directly plated from ice onto plates containing 100ug/mL

ampicillin. If selection of transformants was based on resistance to gentamicin, 1 mL of LB broth without antibiotics was added to the mixture, and the tube and contents allowed to incubate for 45 minutes at 37^0C. This new mixture was then spread onto plates containing 15 ug/mL gentamicin.

2.15 Statistical analyses

Plate counts were analysed with a one-way analysis of variance (ANOVA) table using Prism 2.1 software. Significance of difference between groups was determined using the Newman-Keuls Multiple Comparison test with a confidence interval of 95%. The results of these analyses can be viewed in Appendix I.

3. Results

3.1 Amplification of *gacS* from *P. aeruginosa* genomic DNA

Using the prod 7 primers and PCR parameters described in Table 1, PCR of genomic DNA produced a product, as predicted, of approximately 2 kb in size (Figure 4). Sequencing of this PCR product with prod 7 f/r primers confirmed it's identity as a portion of the *gacS* gene. PCR with MPGACS primers (Table 1) produced a product of approximately 3.4 kb in size as outlined by Parkins (30). No sequence analysis was done on this product, although it was used successfully as template DNA for the prod 7 reaction (results not shown).

3.2 Confirmation of pBSIIgacS

The 2 kb PCR amplification product obtained with the prod 7 primers was blunt-end ligated into pBluescript II KS+ linearized with Eco RV as described in the Methods and Materials section (see Figure 3a). Blue/white screening of transformed E. coli XL1 Blue cells was used to select colonies for plasmid DNA preparation. Isolated plasmid DNA from candidate E. coli XL1 Blue clones showed an increase in size (Figure 5a). Digestion of three candidate clones with Sph I showed linearization of the plasmid at a single point (Figure 5b). Later sequence analysis of the plasmids from these 3 clones using universal primers for pBSII ks+ confirmed the presence of the prod 7 insert.

Figure 4. Gel analysis of PCR of genomic DNA from *P. aeruginosa* PA14 with prod 7 and MPGACS primers.

a.) Prod 7 PCR. Lanes 1-3 correspond to undiluted genomic DNA template, 10x dilution, and 100x dilution, respectively. Lane 4 is a water blank, and lane 5 is 100 bp DNA ladder. The size of the amplification product was approximately 2kb and was verified on other gel runs with a larger size DNA ladder.

b.) MPGACS PCR. Lanes 1-4 correspond to undiluted genomic DNA template, 10x dilution, 100x dilution, and 1000x dilution, respectively. Lane 5 is a water blank, and lane 6 is 100bp DNA ladder. In other gel runs the size of this product was confirmed to be 3.4 kb through the use of DNA ladders with larger size markers.

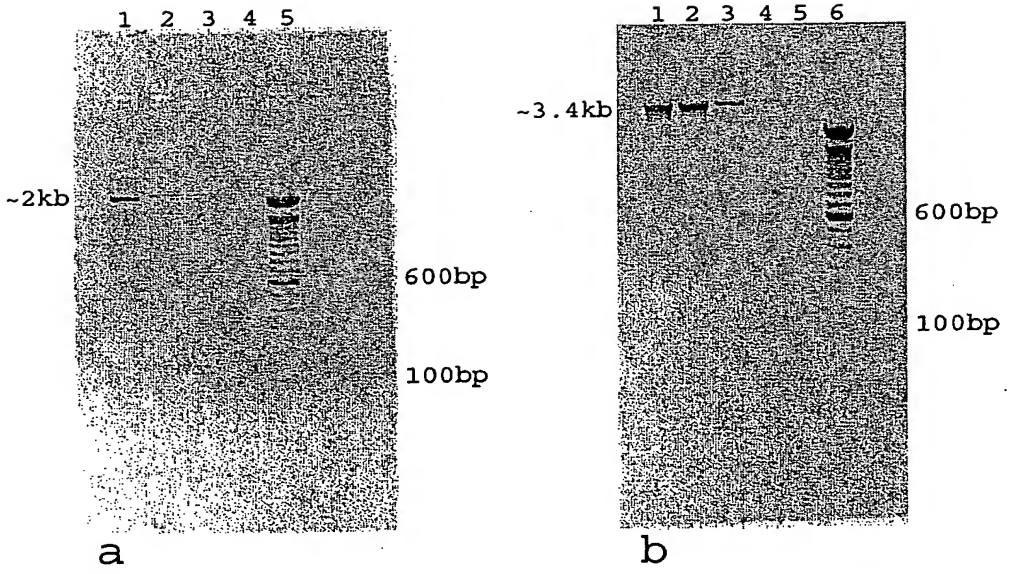
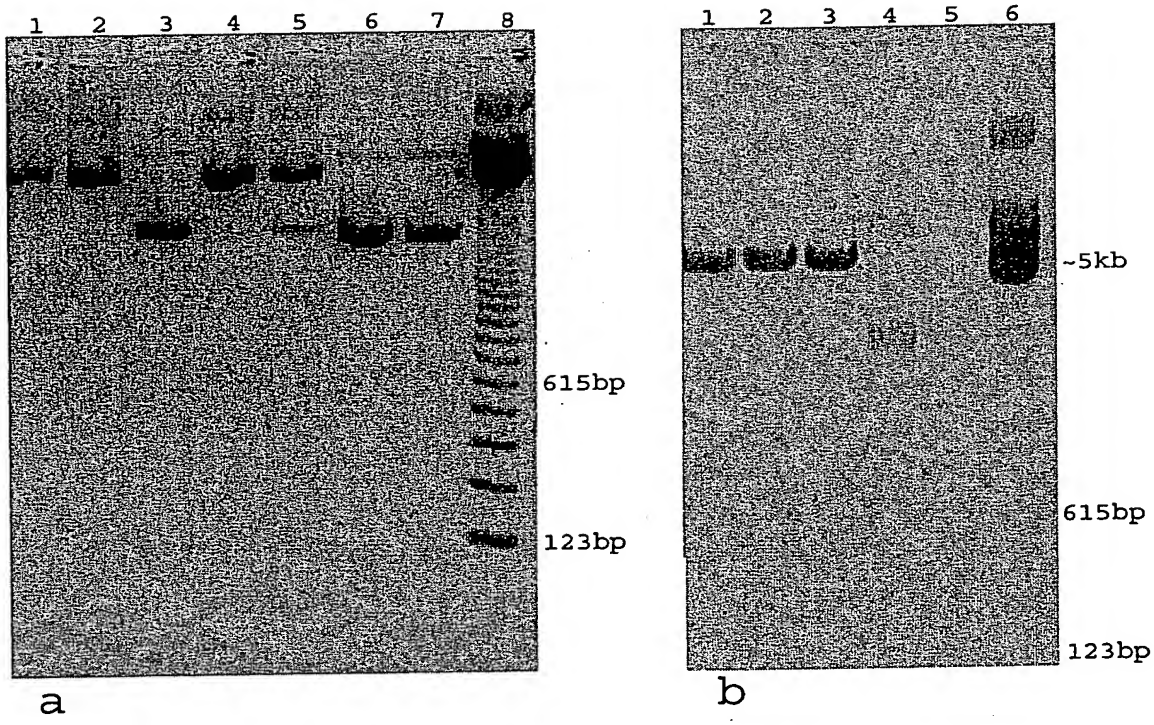

1 2 3 4 5
~2kb
600bp
100bp
a
1 2 3 4 5 6
~3.4kb
600bp
100bp
b

Figure 5. Construction and confirmation of pBSIIgacS.

a.) Minpreps from candidate clones for pBSIIgacS. Note increases in size of plasmids containing inserts in lanes 1, 2, 4, and 5 compared to those lacking inserts in lanes 3, 6, and 7.

b.) Gel analysis of Sph I digestion of minipreps from clones 1, 2, and 4 from Figure 5a (lanes 1-3 respectively) and unaltered pBSII (lane 4). The size of the linearized constructs is approximately 5 kb. Because unaltered pBSII plasmid does not contain a Sph I site, it remains partially supercoiled and resolves as a more diffuse and rapidly migrating band (lane 4).



Attachment D

3.3 Confirmation of pBSIIgacS::gm

Following construction of pBSIIgacS, the recombinant plasmid was cut at a single point within the *gacS* insert with the restriction enzyme Sph I. A gentamicin resistance cassette (gm^R) was isolated following Sph I digestion of the plasmid pUCGM (Figure 6a) and ligated into the Sph I site of pBSIIgacS::gm (see Figure 3b in methods). The aim of this procedure was to introduce a selectable marker, while at the same time, disrupting the *gacS* sequence and inactivation of the *gacS* gene. Constructs with the gentamicin cassette (gm^R) insert were initially identified by the size of the plasmids isolated from candidate *E. coli* JM109 clones (Figure 6b). Further confirmation of the identity of recombinant plasmids was based on Sph I digestion (Figure 6c) and PCR amplification of sequences in the plasmids with prod 7 primers (Figure 6d). Furthermore, candidate clones exhibited gentamicin resistance, indicating a functional gentamicin resistance cassette.

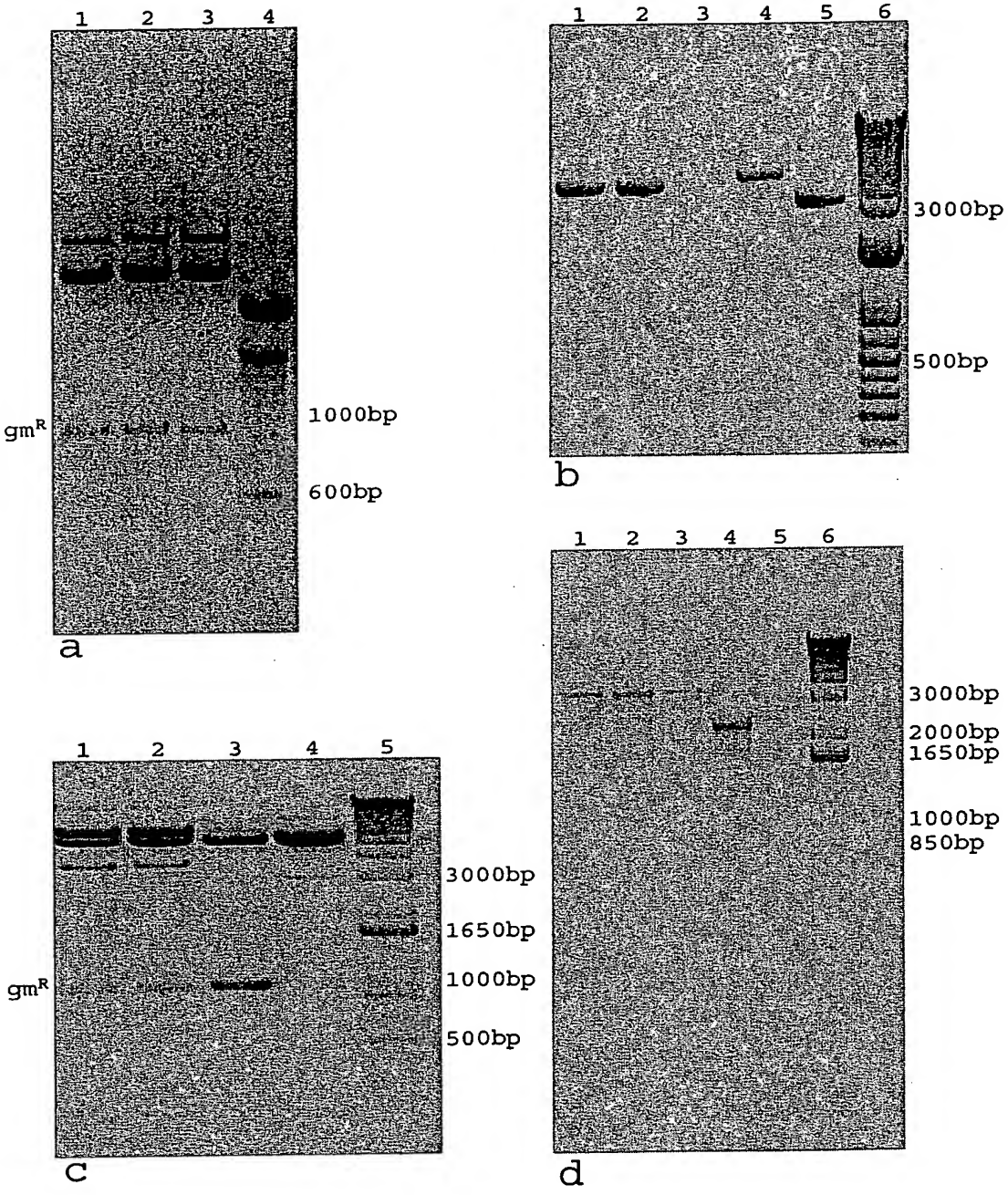
Figure 6. Construction and confirmation of pBSIIgacS::gm

a.) Gel analysis of SphI digestion of pUCGM. The band between 900bp and 1000bp contains the gentamicin resistance cassette. All lanes (except lane 4 which contains a 100 bp ladder) were loaded from the same Sph I digestion. This fragment was gel isolated and used for construction of pBSIIgacS::gm.

b.) Gel analysis of pBSIIgacS::gm minipreps from candidate clones 3 (lane 1),5 (lane2),8 (lane3), 10 (lane 4), and unaltered pBSIIgacS (lane 5). Note that plasmids from candidates 8 and 10 are greater in size than those for clones 3 and 5, suggestive of double inserts.

c.) Gel analysis of incomplete digests of DNA from candidate's 3,5,10, and pBSIIgacS with SphI (lanes 1,2,3, and 4 respectively). The band at approximately 1 kb represents gm^R. The increased intensity of this band in candidate 10 may suggest the insertion of more than one gm^R cassette.

d.) Gel analysis of prod 7 PCR products from candidates 3,5,10, and genomic *P. aeruginosa* PA14 DNA, and water blank (lanes 1, 2, 3, 4, and 5 respectively). Note the increase in size of the PCR product from just over 2kb in the wildtype genomic DNA (lane 4) to approximately 3kb in the recombinant plasmids (lanes 1-3).


1 2 3 4
gm^R
1000bp
600bp
a
1 2 3 4 5 6
3000bp
500bp
b
1 2 3 4 5
gm^R
3000bp
1650bp
1000bp
500bp
c
1 2 3 4 5 6
3000bp
2000bp
1650bp
1000bp
850bp
d

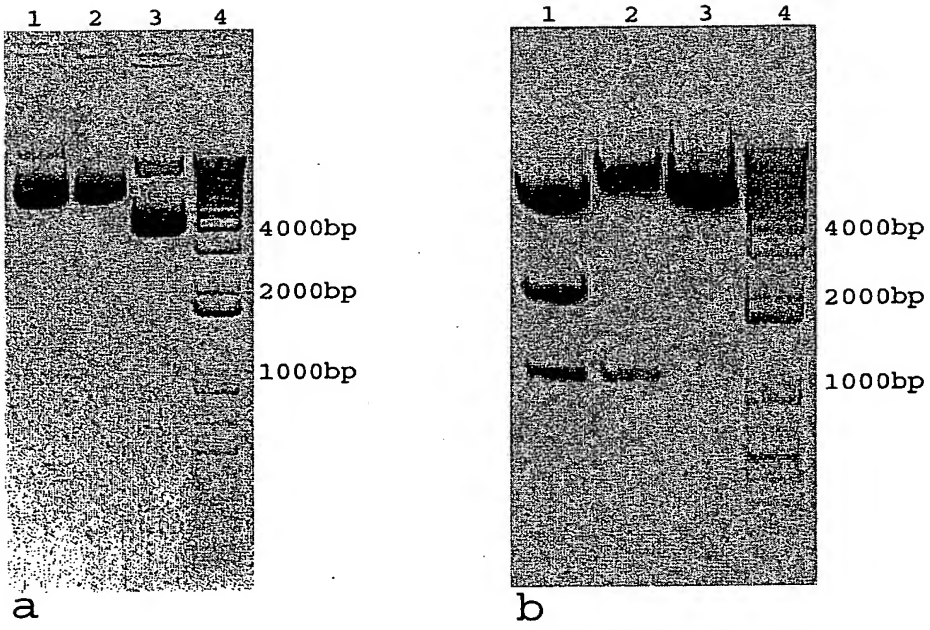
3.4 Confirmation of pEX18gacS::gm

The final recombinant plasmid constructed was made by insertion of the gacS::gm sequence from the Bluescript plasmid into the new vector, pEX18 (Figure 3c) as described in the Methods and Materials section. As in the previous cloning steps, candidate clones of the pEX18gacS::gm construct were first identified on the basis of plasmid size (Figure 7a) and verified by observation of the predicted restriction digestion pattern with Sph I (Figure 7b). Phenotypically, the *E. coli* JM109 clones that carried this construct were resistant to 15 ug/mL gentamicin and were sensitive to 5% sucrose in LB agar. The final selection of clone #2 (Figure 7a) to supply pEX18gacS::gm to *E. coli* SM10 for conjugation with *P. aeruginosa* PA14 was made on the basis of sucrose sensitivity. The selected clone demonstrated more severe sucrose sensitivity than did clone#1. As sucrose sensitivity was one of the phenotypic traits used to distinguish true allelic exchange events from plasmid incorporation, the more severe phenotype was desirable.

Figure 7. Construction and confirmation of pEX18gacS::gm.

a.) Gel analysis of DNA minipreps from pEX18gacS::gm candidate clones 1 and 2 (lanes 1 and 2), unaltered pEX18 (lane 3), and 1kb+ ladder (lane 4).

b.) Gel analysis of Sph I digestion of candidate clones 1, 2, and unaltered pEX18 (lanes 1,2, and 3 respectively). Note the differences in digestion products between candidates 1 and 2. This is accounted for by reverse directions of the gacS::gm insert in clones 1 and 2. Clone 2 was kept for further work as it seemed to show more severe sucrose sensitivity – a desirable characteristic for selection of a true mutant strain.


1
2
3
4
4000bp
2000bp
1000bp
a
1
2
3
4
4000bp
2000bp
1000bp
b

3.5 Sequence and alignment analyses for the *gacS* mutant

Following conjugation of *E. coli* SM10 carrying the pEX18gacS::gm construct with *P. aeruginosa* PA14 and selection of the *gacS* mutants, genomic DNA was prepared and the predicted 3kb PCR product that should be generated with the prod 7 primer set was obtained. This fragment was sent to QIAGEN GENOMICS Inc. Sequencing Services (Bothell, Washington, U.S.A.) to obtain sequence information from each end of the amplified fragment using the prod 7 primers for the sequence read (Figure 8). The sequence obtained was compared to the published genomic *gacS* sequence of *Pseudomonas aeruginosa* PA01 and to the gentamicin resistance cassette sequence of pUCGM. Both ends of the prod 7 sequence reads are within the genomic positions of gacS (1015752 start to 1012975 end) in the PA01 genome. At one end, the first 199 bases of the prod 7 amplified fragment sequence from the gacS- strain share 100% identity with the PA01 gacS sequence. The next 542 bases are greater than 99% identical (542 out of 544) with the published sequence of the pUCGM gentamicin resistance cassette (Figure 8a). These sequence data confirm the disruption of the gacS gene sequence by the gm^R cassette within the mutant. At the other end of the prod 7 fragment, 541 out of 547 bases sequenced are identical (greater than 98%) to the PA01 *gacS* sequence. In terms of the *gacS* region, minor sequence differences may be due to differences between the sequence of *gacS* in PA01 versus PA14. Alternatively, small errors might have been introduced during PCR amplification of the prod 7 product for sequencing. All cloning amplifications were done with a proof-reading polymerase (Platinum Pfx polymerase – Invitrogen), but amplification for sequencing was carried out with conventional *Taq* polymerase, perhaps allowing for

modification. The gentamicin resistance cassette is functional so it is unlikely that any mutation in sequence exists, or if it does, it is not functionally relevant.

3.6 Further confirmation of *gacS* mutant

The candidate clone grew on VBMM and all other media containing 15 ug/mL gentamicin, tolerated 5% sucrose on LB, and died on carbenicillin. Figure 9 a and c demonstrate the amplicons derived from the prod7 and MPGACS amplifications of genomic DNA from candidate mutants. Note the increase in size (by approximately 1 kb) due to the presence of the gm^R cassette in each of the relevant amplicons. Sph I digestion of the prod 7 gacS::gm product produced fragments corresponding to most of 2 kb gacS component and 1 kb gm^R insert (Figure 9b)

Figure 8. Sequence data for gacS::gm, read from the prod7f primer.

a.) Sequence from one end of the inactivated gacS gene containing the gmR insertion. The gmR cassette sequence begins where text colour changes from black to blue.

b.) Sequence data from the other end of the prod 7 amplification product of the gacS gene.

5'-GACATGCGCGCCCAGTTGATCGAGCGCGGGCAACTGATCGCCG
AACAACTGGCGCCGCTGGCCGCCACCGCGCTGGCGCGAAAGGATA
CCGCCGTGCTCAACCGCATCGCCAACGAGGCGCTGGACCAACCGG
ACGTGCGCGCGGTGACCTTCCTCGACGCCCGCCAGGAACGCCTCGC
CCATGCCGGGCCAAGCATGCCTGCAGGTCGACTCTAGAGGATCCC
CGGGTACCGAGCTCGAATTGGCCGCGGCGTTGTGACAATTTACCGA
ACAACTCCGCGGCCGGGAAGCCGATCTCGGCTTGAACGATTTGTTA
GGTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTTCTTCCCGT
ATGCCCAACTTTGTATAGAGAGCCACTGCGGGATCGTCACCGTAAT
CTGCTTGCACGTAGATCACATAAGCACCAAGCGCGTTGGCCTCATG
CTTGAGGAGATTGATGAGCGCGGTGGCAATGCCCTGCCTCCGGTGC
TCGCCGGAGACTGCGAGATCATAGATATAGATCTCACTACGCGGCT
GCTCAAACTTGGGCAGAACGTAAGCCGCGAGAGCGCCAACAACCG
CTTCTTGGTCGAAGGCAGCAAGCGCGATGAATGTCTTACTACGGAG
CAAGTTCCCGAGGTAATCGGAGTCCGGCTGATGTTGGGAGTAGGT
GGCTACGTCTCCGAACTCACGACCGAAAAGATCAAGAGCAGCCCG
CATGGATTTG-3'

a

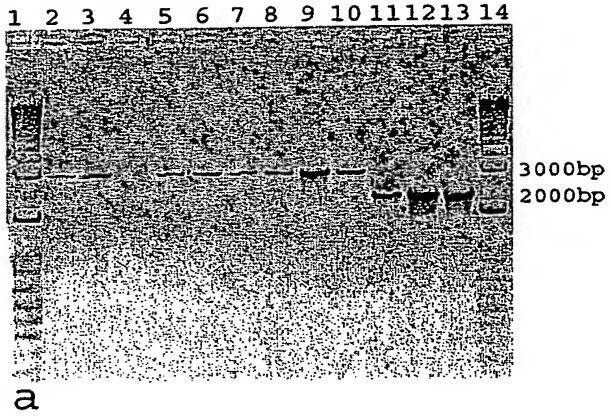
5'-CGCGTTTTTCGCTGACCACCGCGGTGACCTGGGCGCCGAGGTCG
CTGAGCAGGGTCTGCACCAGCAGCAGGTTGGCCGGGTTGTCGTCG
ACGCAGAGCAGCCGAGGCGGCCGTCCGGAAACCATGGCGTGGGGC
TTGTCGCTGCGCGTCGGGCGGACTTGAAGCAACTCCTGCAGCTTGC
GTTGCAGCTTGCGGGTGCAGGCGGGCTTGGCCTCGACCTGTTCGTC
GGGCAGGGTCGCGTGGTATTGCGCCTGCTCGGTGGTCGGGCAGAG
CACCAGGGTCTTGCAGCCGAGCCGTTCGAATTCCCAGAACGACTG
GCTCAGCTCTTCCGGCGGATGGATCGCGGCCGAGACGCCGAGCAC
CGCCAGGCTGATCGGCAACTGGCCGGGCGGCGGGTTGCGCAGGCT
TTCCTGGAGGCTGTCGAGGTCGGCGAATTCGCTCACTTCCAGGCCG
AAGTCGGTGAGCTGGTGGTGCAGCGAGCGGCGCGTCAGCTCCTGC
GGTTCGAGCAGCGCCACGCGCTGGCCCGCGGCCCAGGAGGCGCCT
GGCTCCTCGTTGTC-3'

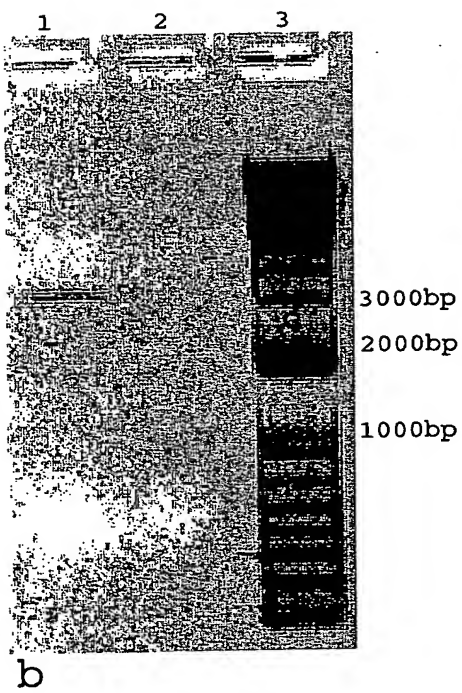
Figure 9. Confirmation of *gacS* inactivation.

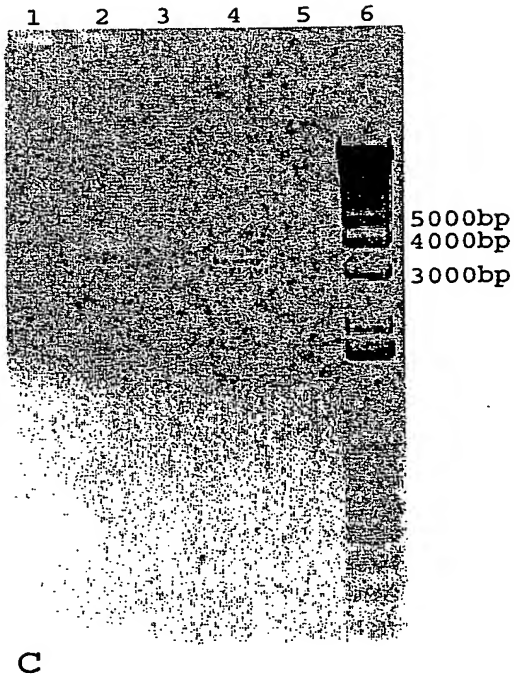
a.) Gel analysis of prod 7 PCR products from candidate *gacS* mutants. Lanes 2-10 are the prod 7 amplicons using genomic DNA from candidate mutant organisms as template. Lanes 11-13 contain amplicons of wildtype P. aeruginosa PA14 DNA. Lanes 1 and 14 contain a DNA 1 kb+ marker ladder.

b.) Gel analysis of Sph I digestion of the prod 7 gacS::gm amplicon seen in lanes 2-10 of panel a. Predicted bands at approximately 1kb (gm^R) and 2 kb (large fragment) are visible in lane 2. Lane 1 contains uncut DNA and lane 3 is a 1 kb+ DNA ladder.

c.) Gel analysis of MPGACS amplicon of gacS::gm. Candidate clones 12, 13, 18 are in the first 3 lanes, followed by wildtype (lane 4) and then a water blank (lane 5). Lane 6 is a DNA ladder. Note the presence of an amplicon of approximately 4.5kb (up from 3.5 kb in the wildtype gene) consistent with the presence of gm^R in the disrupted *gacS* gene. This size fragment could not be produced with these primers except in the circumstance of a double crossover event that transferred the gm^R cassette into the genomic *gacS* gene. If there was direct plasmid insertion, a much larger product would form, if any at all.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
3000bp
2000bp
a


1
2
3
3000bp
2000bp
1000bp
b


1 2 3 4 5 6
5000bp
4000bp
3000bp
c

3.7 Confirmation of pUCP18mpgacS and complemented strains

As phenotypic differences were identified in the mutant strains, it became necessary to further verify that those differences were the result of the inactivation of *gacS*. To this end, the construct pUCP18mpgacS was produced (see Figure 3d in Methods and Materials). This construct was based on the pUCP18 vector which is capable of replicating in *P. aeruginosa* (unlike pEX18) and conferring resistance to carbenicillin through a β-lactamase selectable marker. Into the cloning site of this vector was placed the entirety of the *gacS* gene from *P. aeruginosa* PA14 and its regulating sequences. The MPGACS primer set and PCR parameters were used to amplify the *gacS* gene and approximately 300 bp at either end of the gene via PCR. This reaction was carried out using the proofreading DNA polymerase Platinum Pfx polymerase to ensure that no inactivating mutations were introduced into the gene. Once assembled, this construct was introduced into *P. aeruginosa* PA14 via the same conjugation procedure described in the Methods and Materials chapter. Given that the pUCP18 vector is a medium to high copy number vector, and the *gacS* gene is part of a regulatory system, the pUCP18mpgacS construct was introduced into wildtype *P. aeruginosa* PA14 as a control measure. Any effects of copy number on the phenotype of strains carrying this construct should manifest in this control strain. The identity of this construct was confirmed by the enlargement of the pUCP18 vector and restriction digestion with Sph I. *E. coli* DH5α that carried the construct exhibited resistance to 100 ug/mL ampicillin in nutrient agar through the expression of the β-lactamase selectable marker carried on the vector. They also produced white colonies upon blue/white screening. Once the construct was identified and inserted into *E. coli* SM10, conjugation procedure resulted in 3 separate strains of *P. aeruginosa* complemented

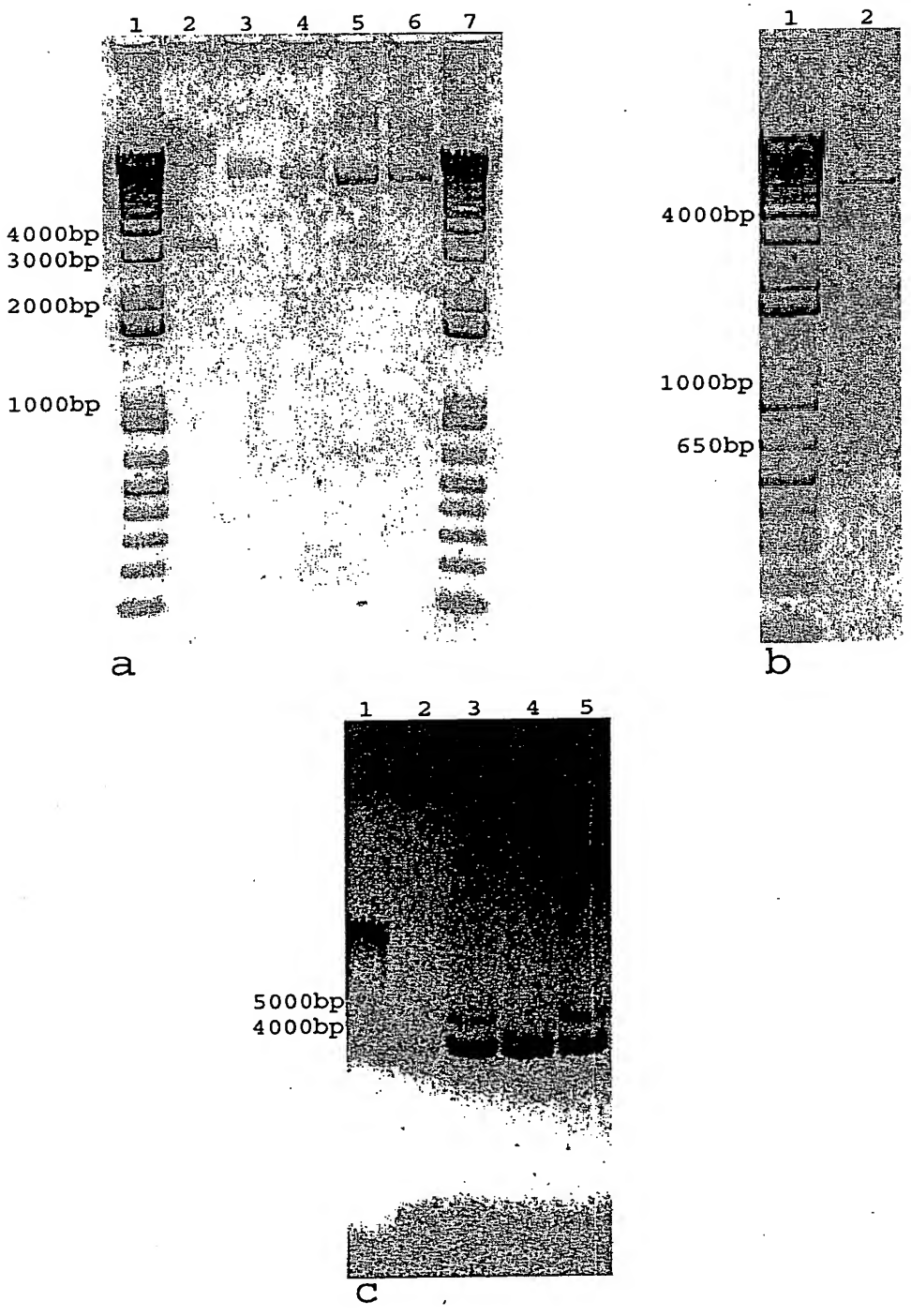
with *gacS in trans* (PA14 (pUCP18mpgacS), GS-N (pUCP18mpgacS), GS-SV (pUCP18mpgacS)). Refer to the later sections of this chapter for an explanation of the nomenclature. PA14 (pUCP18mpgacS), being the complemented version of wildtype P. aeruginosa PA14 was resistant to carbenicillin only. GS-N (pUCP18mpgacS) and GS-SV (pUCP18mpgacS) were resistant to both carbenicillin and 15 ug/mL gentamicin, through the presence of both the pUCP18mpgacS construct and the gm^R cassette inserted into their genomic copy of *gacS*.

Figure 10. Construction and confirmation of pUCP18mpgacS.

a.)Gel analysis of unaltered pUCP18 (lane 2) followed by minipreps from *E. coli* DH5α clones 1,2,4,6 potentially carrying pUCP18mpgacS(lanes 3-6 respectively). Note the increase in size indicative of a successful ligation. Lanes 1 and 7 contain 1kb+ DNA ladder

b) Gel analysis of SphI digestion products of a candidate plasmid from *E. coli* DH5α clone 6 (lane 6 in panel a). Note bands of predicted size of approximately 650 and 7000 bp.

e.) Gel analysis of PCR products from the MPGACS PCR reaction using the total DNA from complemented strains of P. aeruginosa PA14. Lanes correspond to 1 kb+ ladder (lane 1), genomic DNA from wildtype *P. aeruginosa* PA14 (lane 2), GS-SV (pUCP18mpgacS) (lane 3), PA14 (pUCP18mpgacS) (lane 4), and GS-N (pUCP18mpgacS) (lane 5). See sections later in this chapter for an explanation of the nomenclature used. although the ladder is difficult to appreciate, one can see that there are two distinct products in the lanes corresponding to GS-SV (pUCP18mpgacS) (lane 3) and GS-N (pUCP18mpgacS) (lane 5). Significant photo-bleaching of the gel was experienced.


1 2 3 4 5 6 7
4000bp
3000bp
2000bp
1000bp
a
1 2
4000bp
1000bp
650bp
b
1 2 3 4 5
5000bp
4000bp
c

3.8 Biofilms of *gacA* mutant in implant associated infections

To begin to evaluate the importance of the GacS/GacA two-component regulator system on biofilm formation in P. aeruginosa, studies using a *gacA*$^-$ strain on silastic tubing implants in rats were initiated. In the first experiment, the *gacA*$^-$ strain of *Pseudomonas aeruginosa* PA14 produced smaller CFU/implant counts than did the wildtype PA14 as seen in Table 5 ($p<0.001$). It should be noted that in the case of both the PA14 and *gacA*$^-$ innocula, the implant was often found inside small fibrinous adhesions, sometimes in the presence of purulent material. In the case of the sterile tubing, the implants were often difficult to locate as they moved within the abdomen. In cases where the implant could not be found or the animal was euthanized prior to the end of the experiment, the animal and its implants were removed from the data set. Bacteria isolated from the implants grew on PIA, confirming their identity as *Pseudomonas* species in all but one case - a control plate. Figure 11 shows the scanning electron micrographs of *P. aeruginosa* PA14 biofilms on the silastic tubing implants. As a confirmatory measure, the experiment to examine growth of organisms on silastic tubing implants was repeated twice with 5 rats in each group. In neither experiment were the results of the original reproduced. In the first repeat experiment (trial #2 Table 5), no significant differences between any of the groups were found. In the second confirmatory trial, significant differences were found only between the control (saline) implants and the implants exposed to *P. aeruginosa* strains. No significant difference was found between the PA14 and *gacA*$^-$ innocula.

Table 5. Comparison of implant-associated growth (log_{10} CFU/implant) of the *gacA*$^-$ strain of *P. aeruginosa* PA14 against wildtype

Trial #1

	CFU/mL of innoculum
PA14	6.90
gacA$^-$	6.98

replicate #	CNTRL	PA14	gacA-
1	no growth	3.68	no growth
2	no growth	4.72	no growth
3	2.65*	5.18	2.54
4	no growth	5.30	no growth
5	no growth	4.70	no growth
6	no growth	4.70	no growth
7	no growth	4.57	no growth
8	no growth	no growth	no growth
9	no growth	2.65	no growth
10	no growth	6.20	no growth

Trial #2

	CFU/mL of innoculum
PA14	6.65
gacA$^-$	6.70

replicate #	CNTRL	PA14	gacA-
1	no growth	no growth	3.00
2	no growth	4.74	no growth
3	no growth	2.18	4.08
4	no growth	removed	no 4.51
5	no growth	removed	no growth

Trial #3

	CFU/mL of innoculum
PA14	6.48
gacA$^-$	6.92

replicate #	CNTRL	PA14	gacA-
1	no growth	4.26	no growth
2	no growth	removed	removed
3	no growth	2.08	5.34
4	no growth	5.36	2.63
5	no growth	4.72	3.91

* these colonies were shown to be contaminant organisms

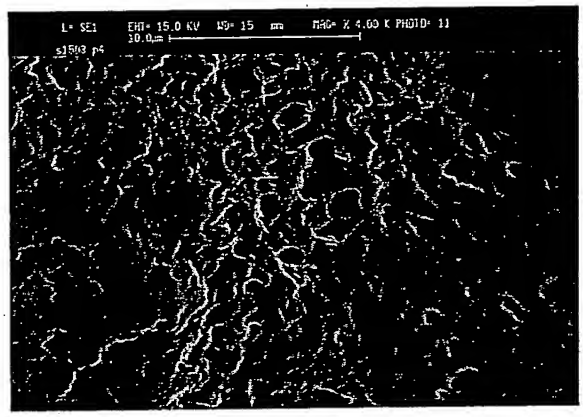
Figure 11. Scanning electron micrographs of *P. aeruginosa* PA14 biofilms on silastic tubing implants formed in the abdominal cavity of rats.

a.) A biofilm of classical morphology. Note that while individual cells are distinguishable, many are mostly or completely encased within the EPS.

b.) This represents a biofilm more typical of implant-associated infections. Few bacteria are obvious and there is much material that may be of host origin (e.g. fibrin, inflammatory debris, etc)

c.) A relatively low magnification image of a PA14 biofilm. Note that the biofilm is a sporadic feature on the surface of the tubing/implant.

d.) This piece of tubing was removed from a purulent lesion in the abdomen. The surface is caked with host cells, and no bacterial cells are distinguishable.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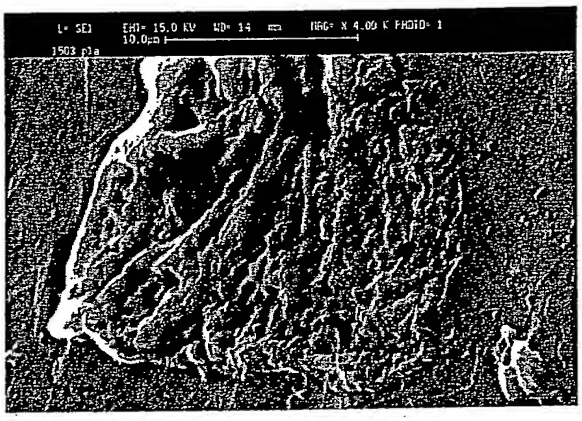
10.0µm
s1503 p4

a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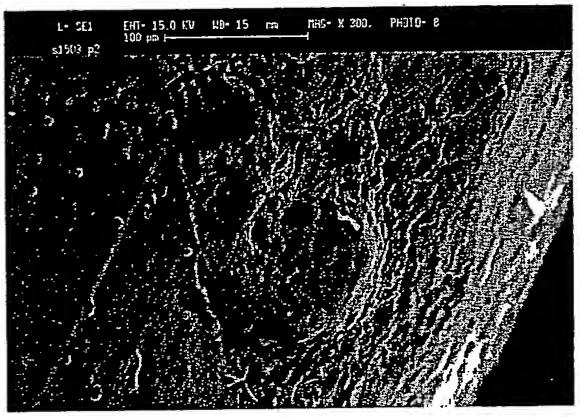
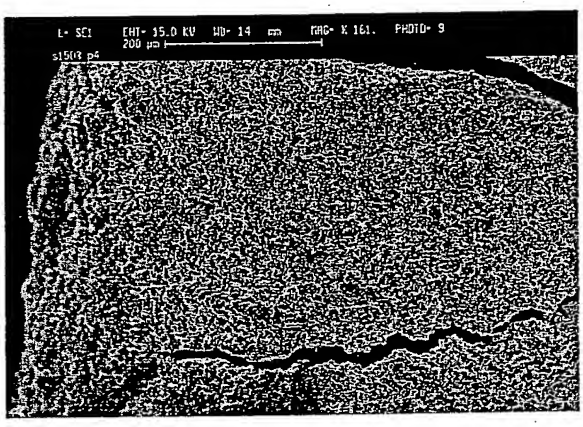
10.0µm
1503 p1a

b


L= SE1 EHT= 15.0 KV WD= 15 mm MAG= X 300. PHOTO= 8
100 µm
s1503 p2

c


L= SE1 EHT= 15.0 KV WD= 14 mm MAG= X 161. PHOTO= 9
200 µm
s1503 p5

d

3.9 Planktonic and biofilm growth curves of PA14 vs. *gacA*⁻ vs. *gacS*⁻

As outlined in methods, one of the phenotypic parameters evaluated for the *gacS* mutant was the rate of planktonic versus biofilm growth of the *gacS* mutant in comparison to the *gacA*⁻ strain and wildtype PA14. No differences in planktonic or biofilm growth were noted between strains (Figure 12).

3.10 Planktonic and biofilm growth curves of PA14 vs. GS-N vs. GS-SV

It was noted that *gacS*⁻ cultures generated small colony variants – especially when the initial innoculum was greater than 24 hrs old and in the later stages of the curve. These small variants were stable in culture (45 days serial plating, prolonged liquid culture) and seemed more prevalent in biofilm samples (data not shown). With this finding, the *gacS*⁻ strain was split into two designations. GS-N designates *gacS*⁻ *P. aeruginosa* PA14 that forms normal looking colonies on nutrient agar (Figure 14). The growth curve data labelled as *gacS*⁻ in Figure D1 can be thought of as GS-N data. GS-SV denotes the phase variant generated from the *gacS*⁻ (GS-N) parent strain. GS-SV (small variant) colonies are smaller than their GS-N counterparts (Figure 14).

The small variant, when grown in a CBD, showed increased biofilm production and a propensity to "slough" as the experiment progressed and the films became more massive (Figure 15). When large sample sets involving 8 or more pegs were analyzed at 11 or 20 hours, statistically significant increases ($p<0.001$) in biofilm production in the GS-SV strain were observed (Appendix I Tables 11-14). GS-SV biofilms were always the highest in CFU/peg compared to the other strains (Figure 13). The planktonic

numbers of GS-SV were not significantly different from PA14 but were significantly lower than GS-N at two time points (11 and 20 hrs. Appendix I Tables 15 and 16). The films produced by GS-SV were also visibly opaque compared to those produced by PA14 and GS-N (Figure 16). A significant difference did exist between PA14 and the complemented PA14 (pUCP18mpgacS). The presence of antibiotics and a resistance gene had little to no effect on planktonic populations, while biofilm populations are lower in the complemented strain by almost 1 $\log_{10}$ unit.

Figure 12. Planktonic and biofilm growth curves of normal PA14, *gacA*⁻, and *gacS*⁻. Two separate pegs or broth culture samples were used for each data point and each of these was plated twice to obtain an average CFU/peg or mL of culture.

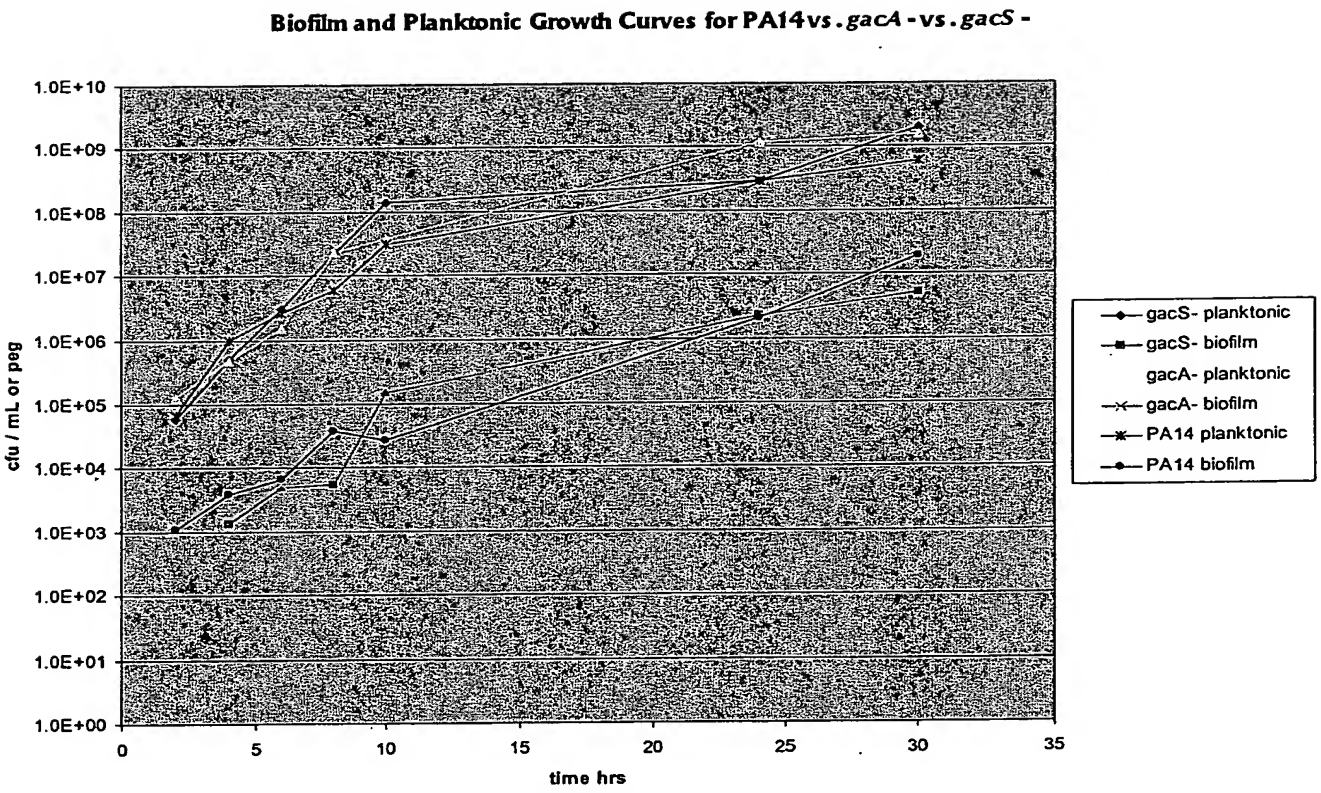
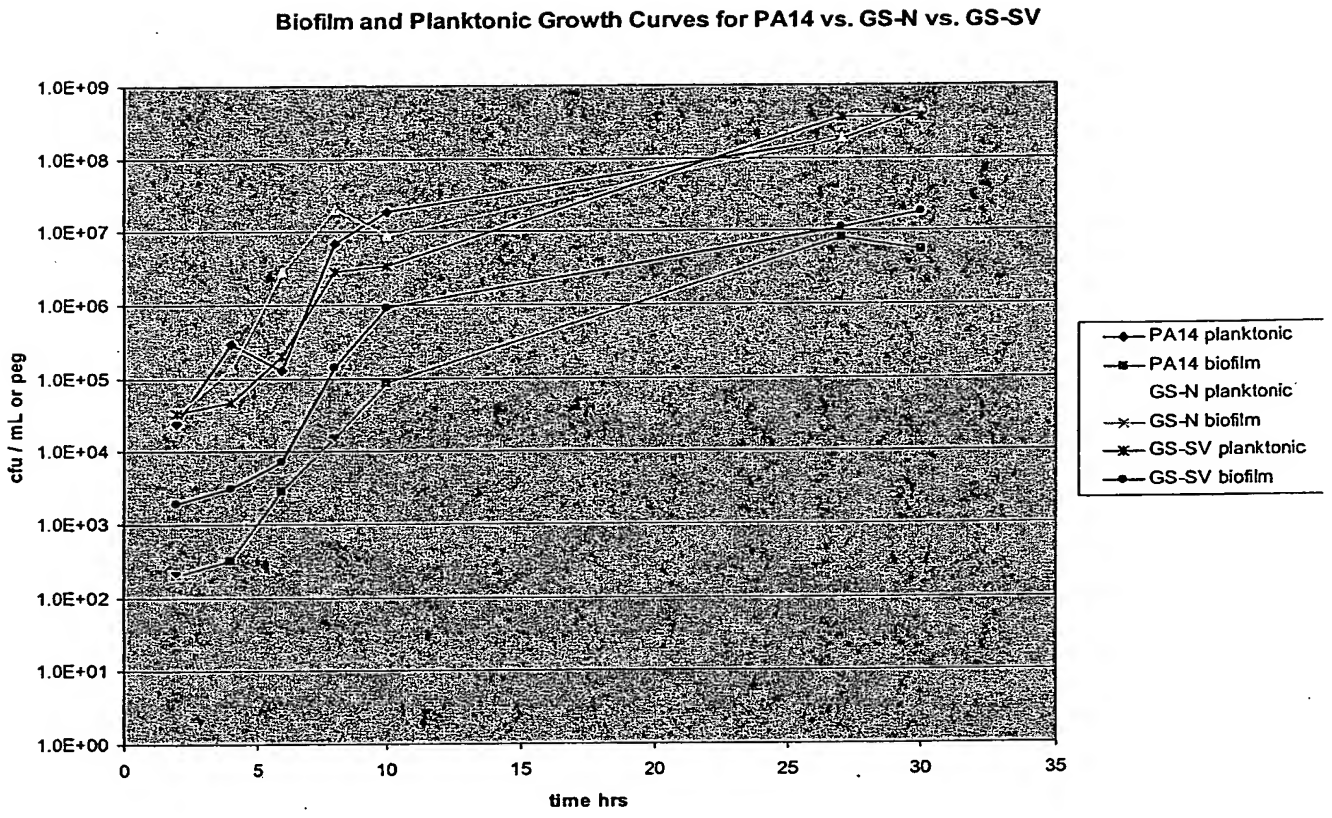
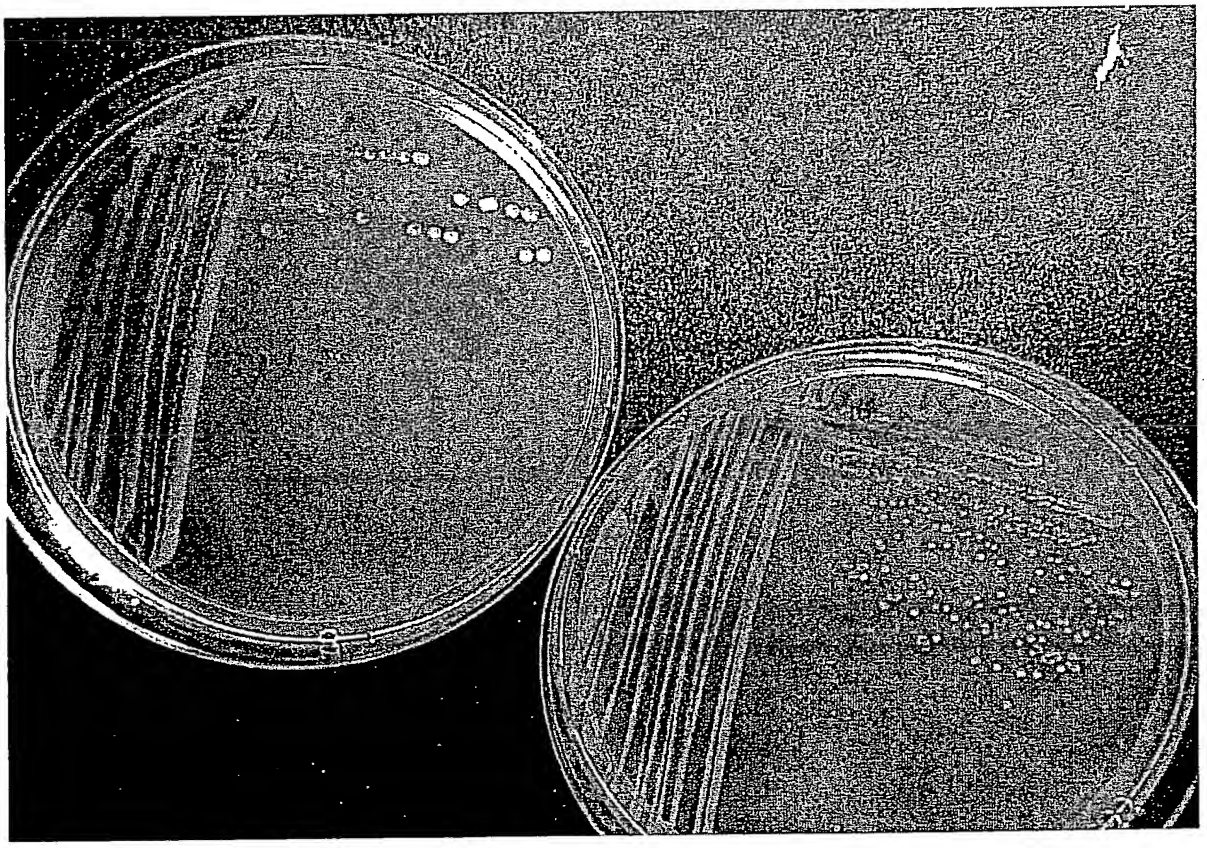

Biofilm and Planktonic Growth Curves for PA14vs.gacA-vs.gacS-
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
cfu / mL or peg
0
5
10
15
20
25
30
35
time hrs
gacS- planktonic
gacS- biofilm
gacA- planktonic
gacA- biofilm
PA14 planktonic
PA14 biofilm

Figure 13. Planktonic and biofilm growth curves of normal PA14, GS-N and GS-SV. Note that GS-SV shows higher CFU counts on biofilm samples despite planktonic numbers that are often the lowest of the three strains.



Attachment D

Figure 14. Appearance of GS-N (upper left), and GS-SV (lower right) colonies on nutrient agar incubated overnight at 37^0C.



Attachment D

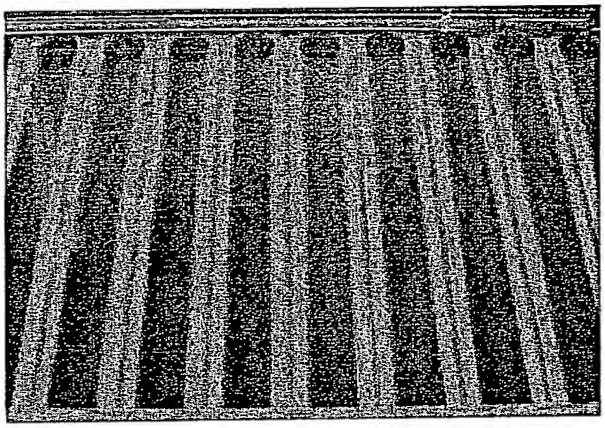
Figure 15. Appearance of broth cultures of PA14, GS-N, and GS-SV. Photographs of TSB broth culture were taken after generation of biofilms in a CBD for 21 hours. When agitated, PA14 broth cultures appeared to have a green hue, while GS-N and GS-SV cultures seemed light yellow.

a.) PA14

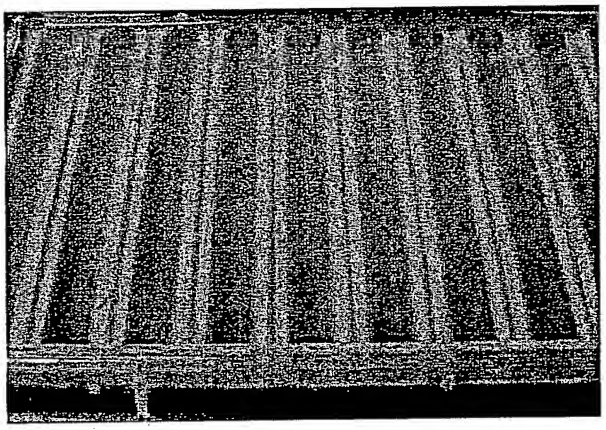
b.) GS-N

c.) GS-SV. Note the flocculant material (likely sloughed biofilm) in the troughs.

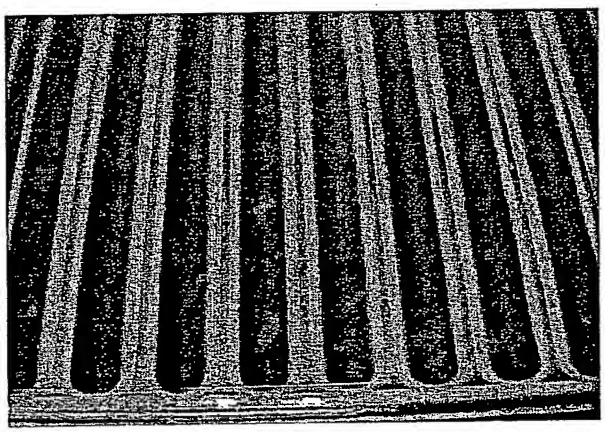
a.



b.



c.



Attachment D

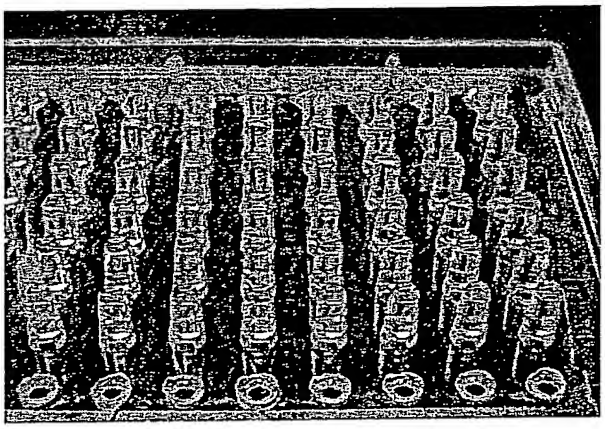
Figure 16. Photographs of biofilms of PA14, GS-N, and GS-SV. Peg lids from a CBD were photographed after 21 hours of incubation in TSB. Note the variability within and between rows. Variability in biofilm size between rows is likely the result of differing "high water marks". Variability within rows is likely the result of some sloughing of the films.

a.) PA14.

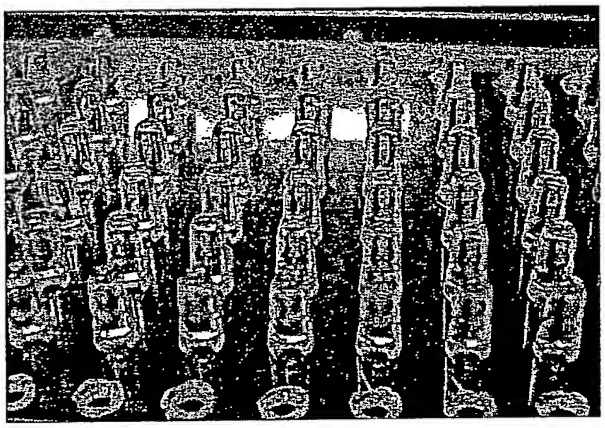
b.) GS-N.

c.) GS-SV. Note the almost complete opacity of the films.

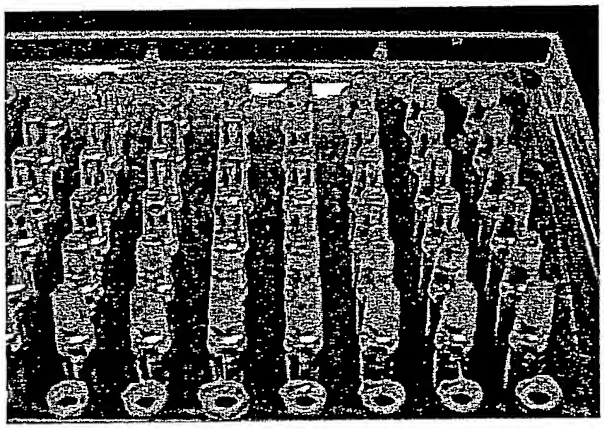
a.



b.



c.

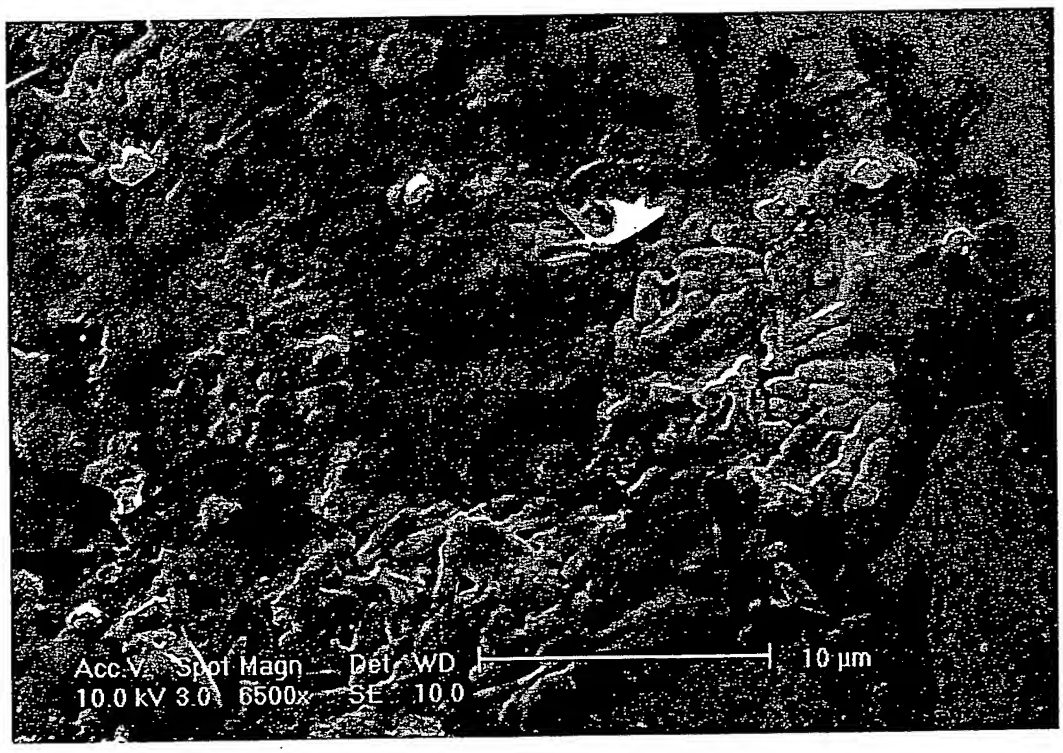


3.11 Structure of biofilms as assessed by scanning electron microscopy

Scanning electron micrographs were taken of pegs collected during the course of the PA14 vs. GS-N vs. GS-SV growth curve experiment. These micrographs showed marked differences in biofilm development. GS-SV produced detectable films at an earlier stage than did the other strains with the first visible biofilms being present as early as 4 hours of incubation (Figure 17). These films had relatively little structure but by 8 hours of culture, the GS-SV biofilms were significantly increased in size with a more organized structure (Figure 18b). At the same stage of culture (8 hours), individual cells were present on GS-N pegs but no structures resembling biofilms were found by scanning electron microscopy. With the wildtype strain, PA14, the first biofilms were detectable at 8 hours, but these were rare and very small (Figure 18a). Thus, at 8 hours of culture, GS-SV films were clearly at a more advanced stage of development than were the films of PA14 or GS-N. This is even better illustrated in the low power scanning electron micrographs of PA14, GS-N, and GS-SV pegs at 10, 27, and 30 hours of culture (Figure 19). While the 10 hour GS-SV biofilm covers much of the peg (Figure 19c 10 hrs.), biofilms are still virtually undetectable on the PA14 and GS-N pegs at the same time point (Figures 19 a and b 10 hrs.). At higher magnification, the PA14 biofilms at 10 hours are still rare and small (Figure 20a) whereas the GS-SV biofilm is showing signs of true structure and organization (Figure 20b). The differences in appearance of PA14, GS-N, and GS-SV biofilms grown on pegs in the CBD, when examined by scanning electron microscopy, continued to be very distinctive even at the longer periods of incubation of 27 and 30 hours toward the end of the experiment. At lower magnification, the density of the biofilm on pegs continues to be greatest for GS-SV (Figure 19, 27hrs,

and 30 hrs.). There is a bumpiness on the surface and the film shows cracking and curling due to dessication artefact that only occurs with the thickest of films. Such a dessication artefact also is detectable in the low power image of the PA14 peg at 30 hours (Figure 19a, 30 hours) but it is still less marked than in the GS-SV peg at the same culture interval (Figure 19c, 30 hrs.). The higher magnification scanning electron micrographs further demonstrate the differences between the various biofilms at 27 to 30 hours with the most distinguishing feature being the marked increase in organization and thickness of the GS-SV biofilms compared to PA14 and GS-N (Figures 21 and 22).

Figure 17. Scanning electron micrograph of a GS-SV peg at 4 hrs. Note the relative lack of structure. These immature films were found at the air/fluid interface and were quite rare.

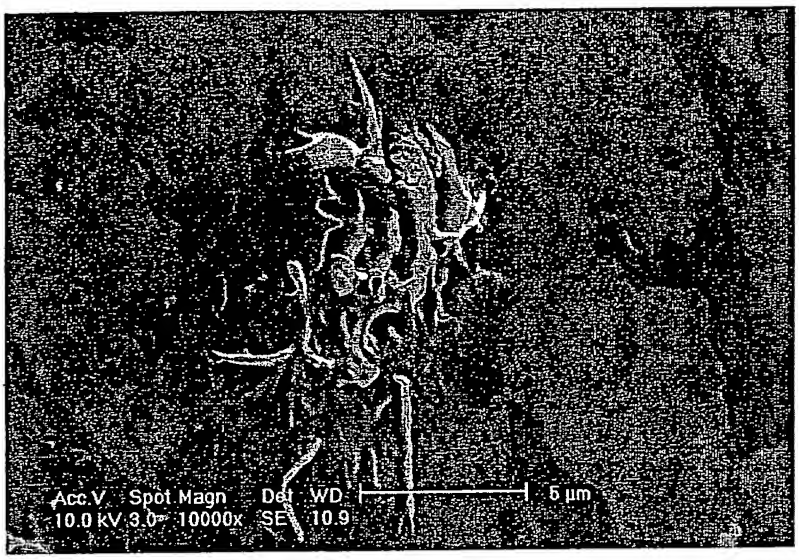


Attachment D

Figure 18. Scanning electron micrographs of PA14 and GS-SV biofilms at 8 hours of culture.

a.) PA14 peg. The first biofilms detected for PA14 were at 8 hours and were rare and small.

b.) GS-SV peg. At 8 hours, GS-SV biofilms showed markedly increased size and structure compared to PA14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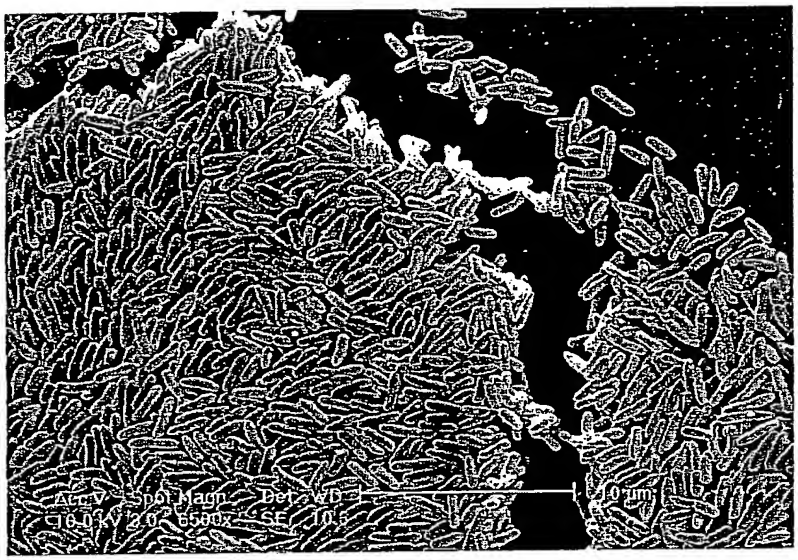
10.0 kV 3.0 10000x SE 10.9
5 µm

a


Acc.V Spot Magn Det WD
10.0 kV 2.0 6500x SE 10.5
10 µm

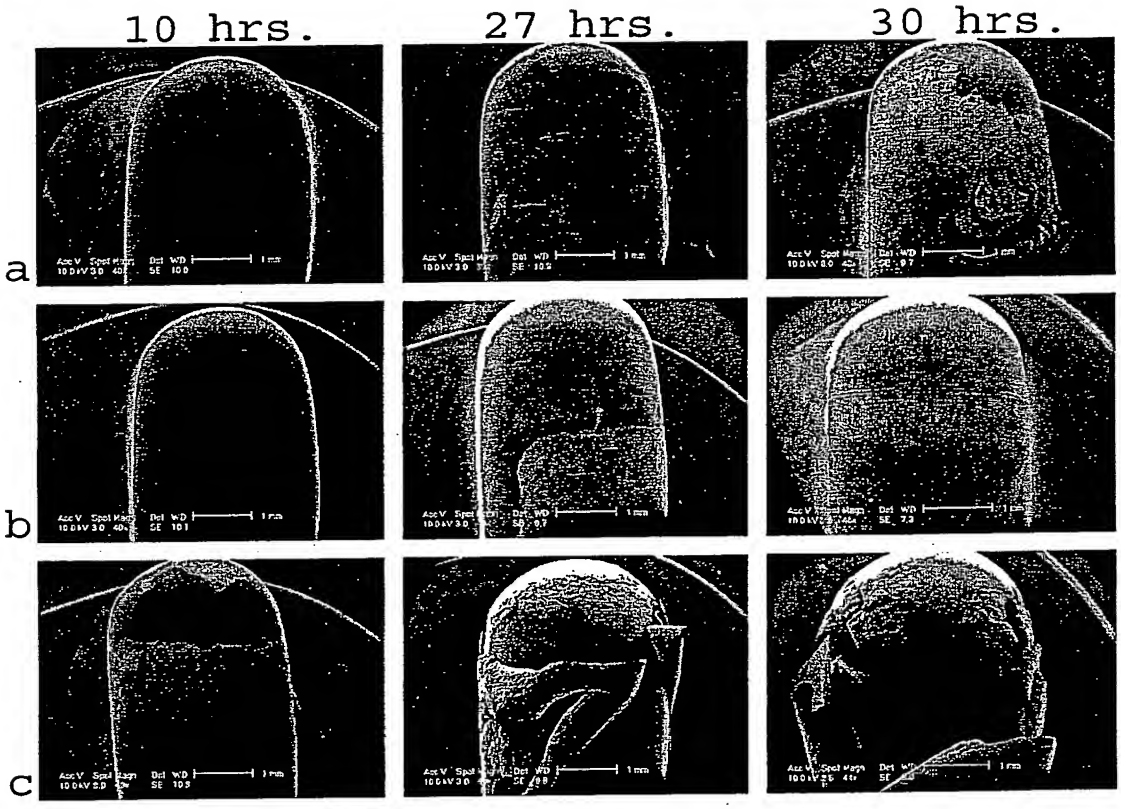
b

Figure 19. Low magnification scanning electron micrographs of PA14, GS-N, and GS-SV biofilms at 10, 27 and 30 hours of culture. Rows are identified with letters and represent individual strains. Each column represents a given time point.

a.) PA14. Little biofilm coverage at 10 hours with increasing spread and depth with time.

b.) GS-N. Little biofilm coverage at 10 hours. Though spread of attached organisms increases, the level of biofilm development never approximates that of the other two strains.

c.) GS-SV. Most of the peg is covered with what appears to be a biofilm even at 10 hours. There is increased thickening of the biofilm at 27 hours with obvious surface bumpiness. These features remain largely unchanged at the 30 hour timepoint. There is significant dessication artefact as a result of the thickness of the film.

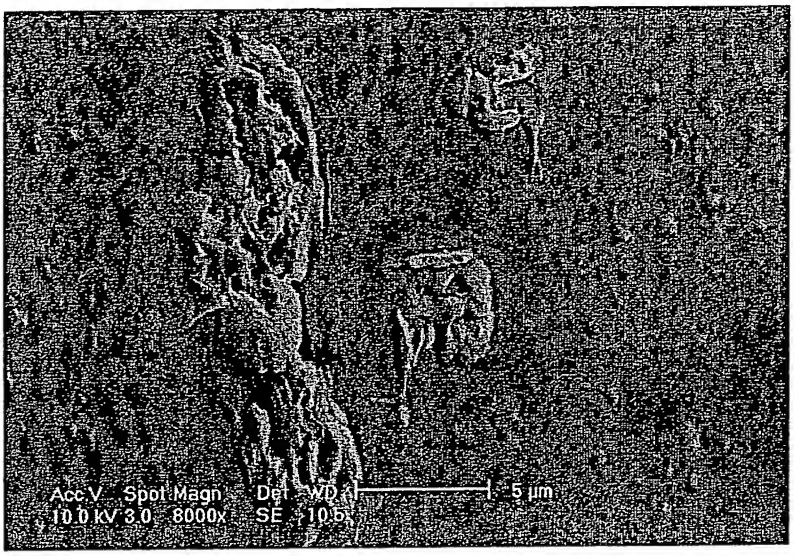


Attachment D

Figure 20. Scanning electron micrograph of PA14 and GS-SV biofilms at 10 hours of culture.

a.) PA14 biofilms.

b.) GS-SV biofilms.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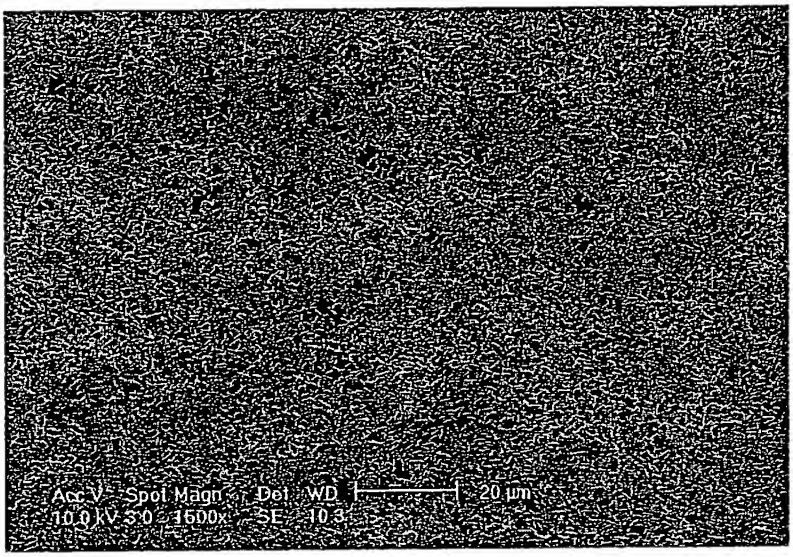
a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10.0 kV 3.0 1500x SE 10.3

b

Figure 21. Scanning electron micrographs of PA14, GS-N, and GS-SV biofilms at 27 hours of culture.

a.) PA14 biofilm.

b.) GS-N biofilm.

c.) GS-SV biofilm. Note how much less distinguishable the cells are in this biofilm compared to those from the other two strains.

d.) Another image of the GS-SV biofilm that demonstrates the thickness of the film in some places. Note the underside is also visible.

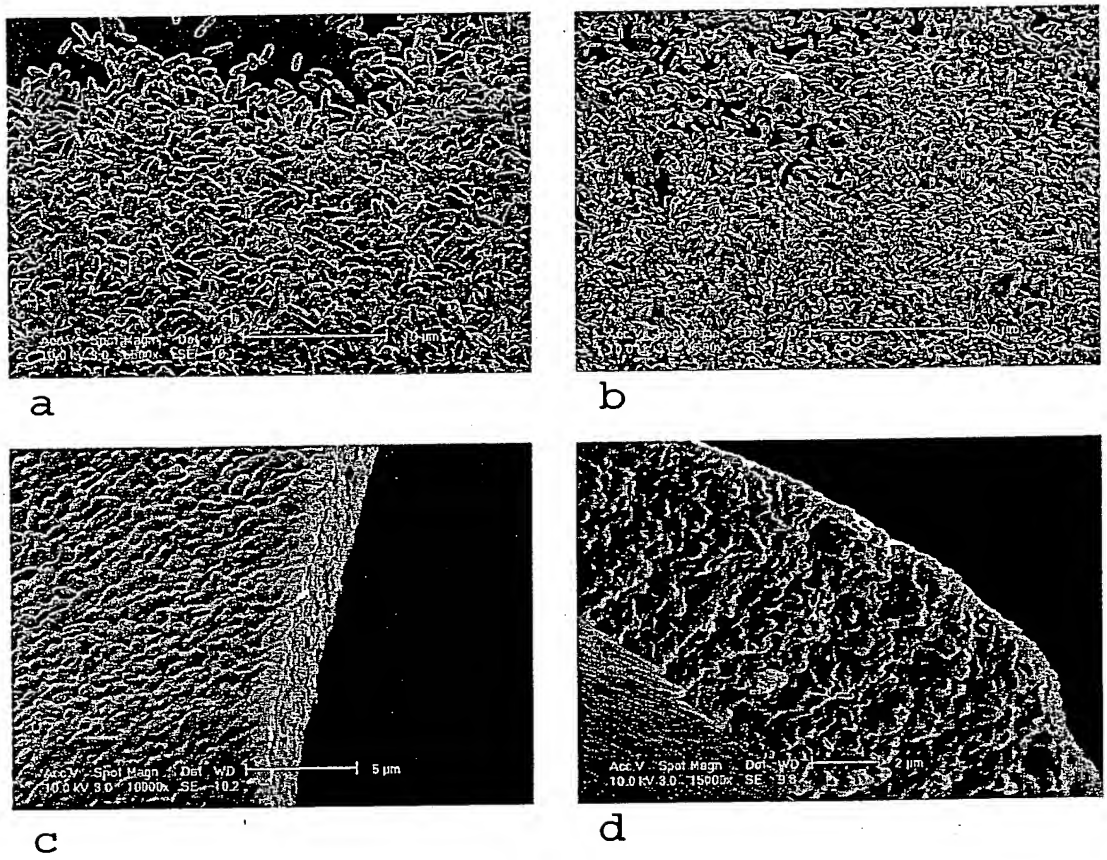

a
b
c
d

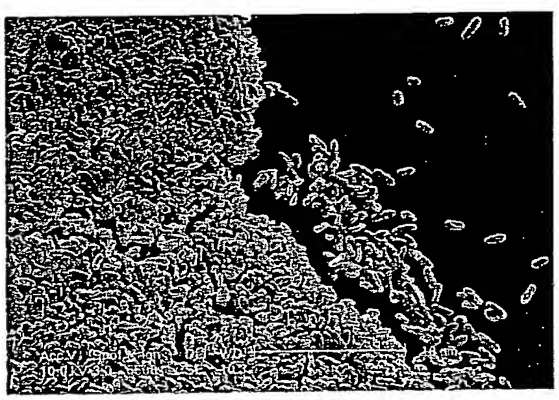
Figure 22. Scanning electron micrographs of PA14, GS-N, and GS-SV biofilms at 30 hours of culture.

a.) PA14 biofilm.

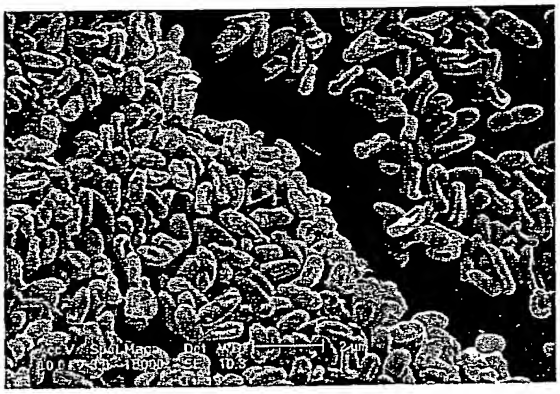
b.) higher magnification of the image in a. Note the puffed wheat appearance of the cells. This is likely part of the dessication artefact inherent to this type of imaging.

c.) GS-N biofilm at 30 hrs. Note that this biofilm is still relatively disorganized compared to the PA14 and GS-SV biofilms.

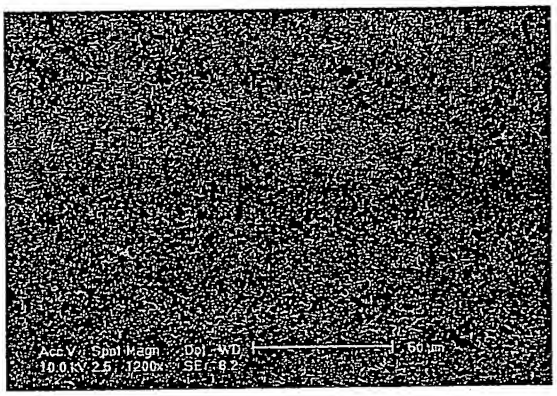
d.) GS-SV biofilm



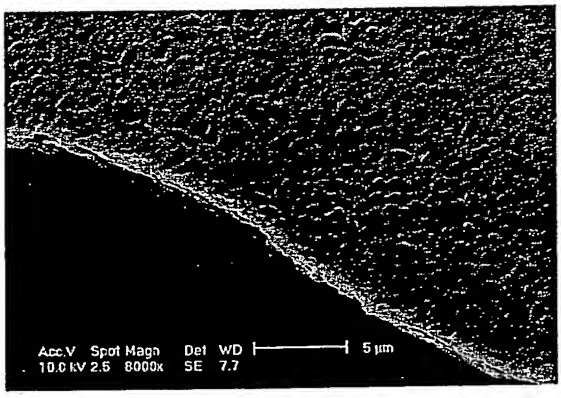
a



b


Acc.V Spot Magn Det WD
10.0 kV 2.5 1200x SE 8.2
50 µm

c


Acc.V Spot Magn Det WD
10.0 kV 2.5 8000x SE 7.7
5 µm

d

In an attempt to reduce dessication artefact and to obtain images that better demonstrate the extracellular matrix material present in biofilms, more CBD pegs were processed for environmental scanning electron microscopy (ESEM). In this mode, samples are viewed without first coating them with gold or paladium in a chamber containing a small amount of water vapour. As such they avoid the vacuum dessication inherent to the sputter coating process. However, these images are inherently 'fuzzier" than those generated by standard techniques. Furthermore, 3 pegs from each strain at a single time point were imaged in this and the following electron microscopy studies so as to minimize the effects of inter-peg variability.

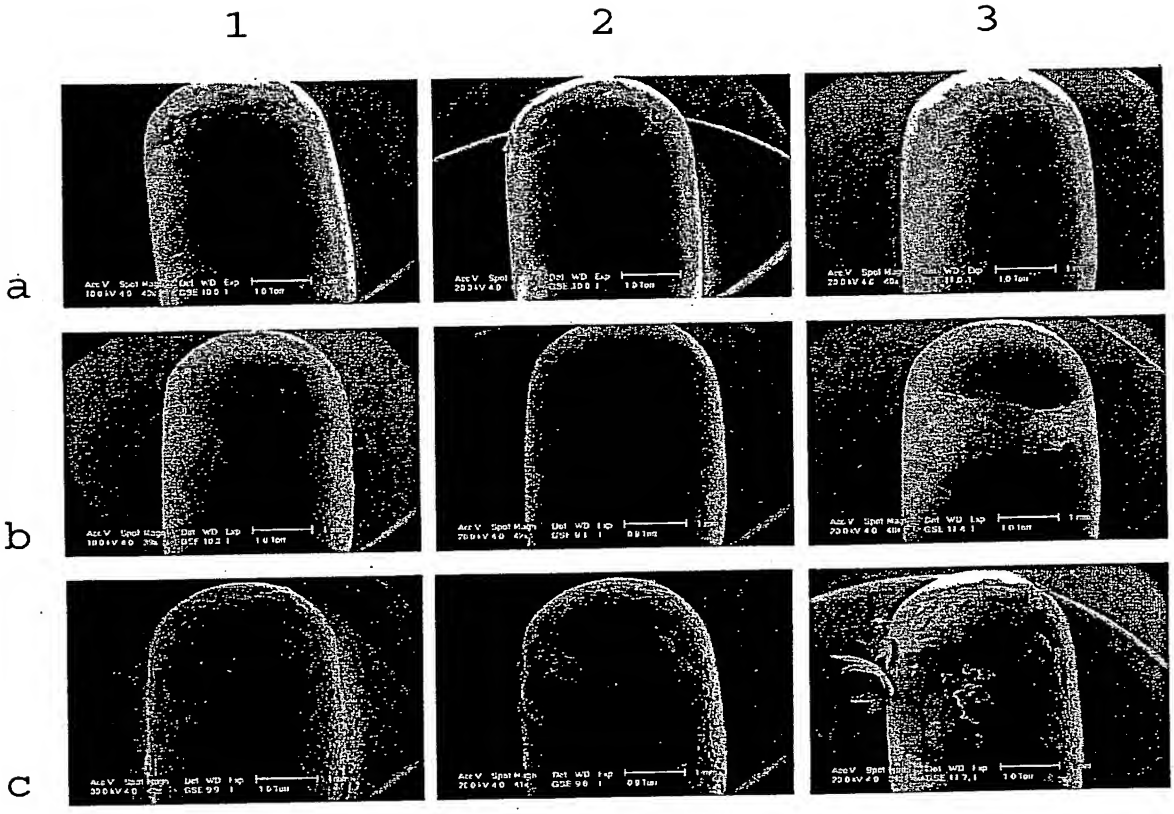
In pegs collected at 24 hrs and viewed under ESEM mode, more differences in biofilms between PA14, GS-N and GS-SV became apparent. PA14 produced largely uniform biofilms with distinct cells. All three of the pegs were almost interchangeable in appearance. GS-N produced at best a loosely organized mass of cells that may or may not be a biofilm. More often than not with GS-N, a reticulated pattern with little structure was observed. Some spots of structure were visible but they were few and far between. Finally, the films produced by GS-SV were very thick and had more severe topography than either of the other two. Although the "bumpiness" observed may be a result of procedures used for scanning electron microscopic analysis, the results strongly suggests that the structures of these films are different from those for GS-N and PA14.

Figure 23. Low magnification ESEM photomicrographs arranged to produce an overview of pegs collected at 24 hr for imaging. Letters refer to strains. Numbers in columns simply refer to individual images on different pegs.

a.) PA14. Note the similar structure and coverage of the films. Defects at the tip are likely the result of contact with the inside surface of a microfuge tube during fixation.

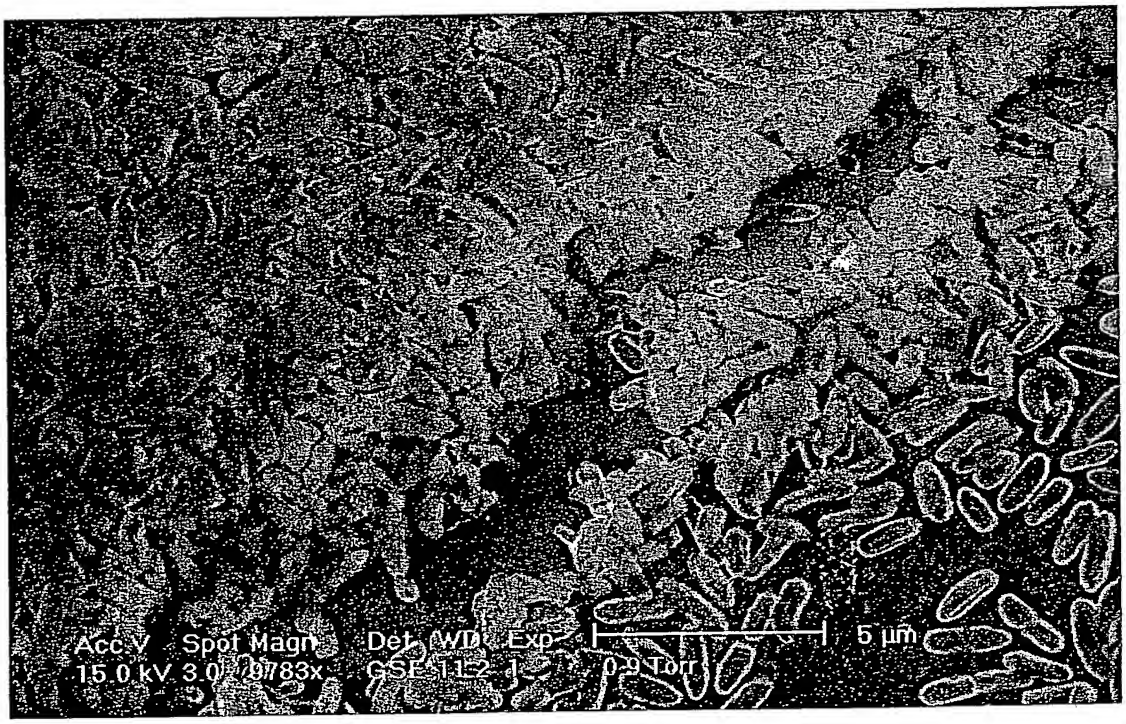
b.) GS-N. Notice that only the last peg (b3) shows any substantial coverage.

c.) GS-SV. Note substantial dessication artefact (cracking) and the bumpy appearance of the surface of the film occur at approximately the same time point.



Attachment D

Figure 24. Environmental scanning electron micrograph of PA14 biofilm at 24 hours. ESEM produces images that are somewhat different in appearance from those of standard SEM.



Attachment D

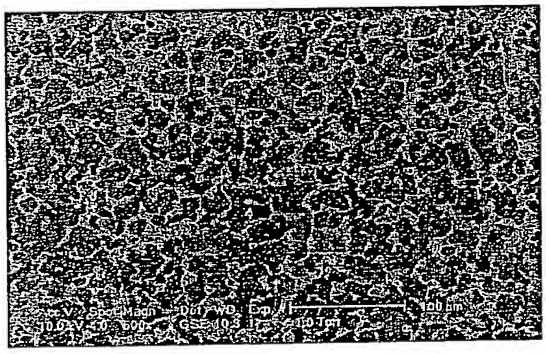
Figure 25. Typical biofilm structures from GS-N pegs at 24 hours of culture as revealed by environmental scanning electron microscopy.

a.) Bacterial cells on peg b1 (from Figure 23) are distributed in a monolayer adopting a reticulated pattern.

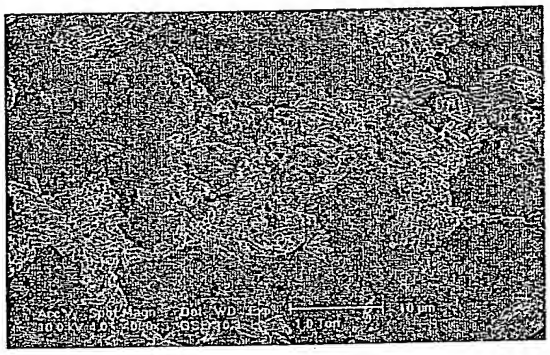
b.) A higher magnification view of the same region as shown in a.

c.) Similar structures were found on peg b2 (from Figure 23).

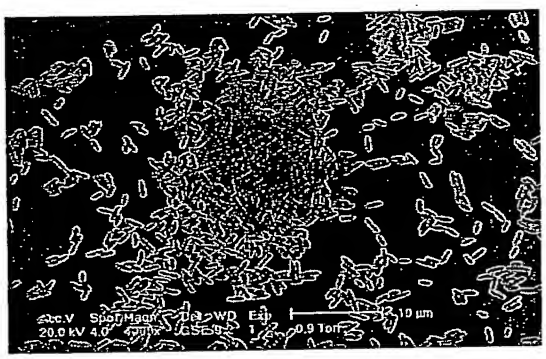
d.) Biofilm structures from GS-N peg b3 (from Figure 23). Note that despite a greater number of cells, there is little organized structure. Crystals in the Figure are known to contain large amounts of arsenic (from x-ray microanalysis), and are therefore assumed to be crystalized cacodylate from the fixation buffer used in the preparation of these samples.



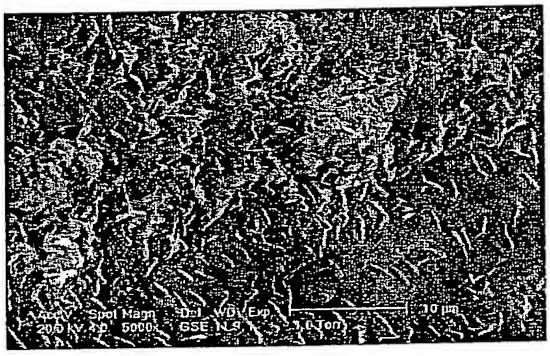
a



b



c



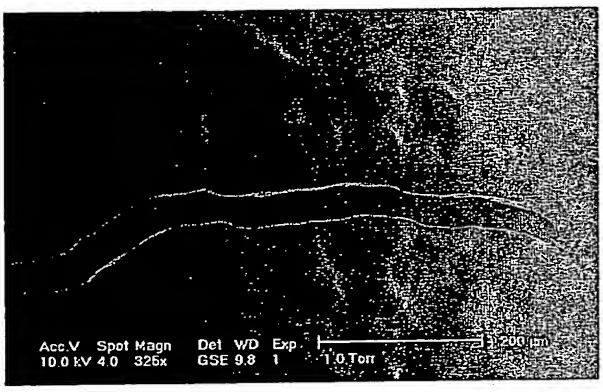
d

Figure 26. GS-SV films at 24 hrs imaged under ESEM.

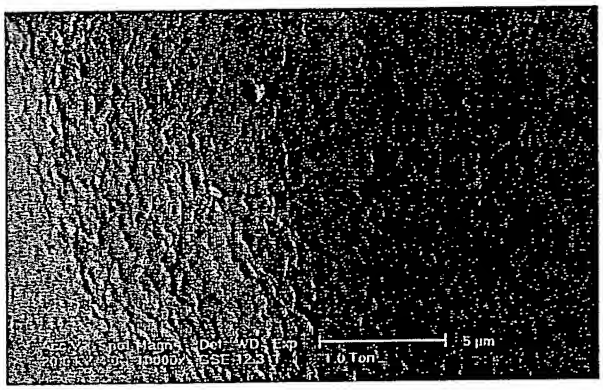
a.) Higher magnification view of the surface of peg c1 (from Figure 23). This image demonstrates the bumpy surface of the film. The areas of depression and elevation would appear to be reasonably regular in their interval. One can also see that the film actually varies in thickness. Thus, the bumps on the surface of the film are unlikely to be the result of the film lifting off its substrate.

b.) The surface of GS-SV biofilms. Note that the cells are still definable, but less so than with films from either of the other two strains.

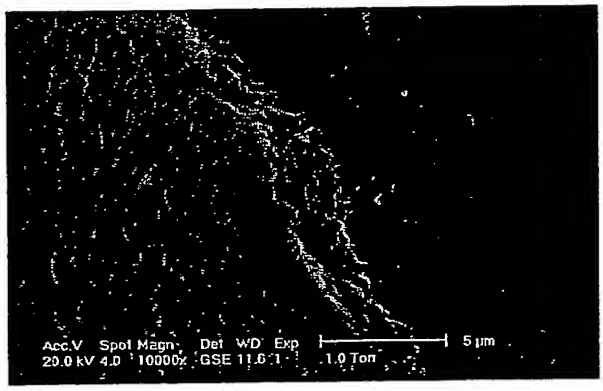
c.) The edge of a crack formed through dessication shows how deep the films from GS-SV can be in some places.


Acc.V Spot Magn Det WD Exp
10.0 kV 4.0 325x GSE 9.8 1 1.0 Torr
200 µm

a


5 µm
1.0 Torr

b


Acc.V Spot Magn Det WD Exp
20.0 kV 4.0 10000x GSE 11.6 1 1.0 Torr
5 µm

c

Figures 27-30 show pegs collected at 26 hrs from complemented strains. These ESEM images were collected to verify that the changes seen in previous photomicrographs were indeed the result of *gacS* inactivation and not due to other factors. Specifically, these studies were designed to confirm the characteristics of the GS-N strain. The GS-SV strain was of less interest as it has a number of altered phenotypic traits, some of which may be due to *gacS* inactivation, while others may be due to phase variation. One notes that the proportion of the peg covered by an at least partially organized biofilm is increased (on average) when the GS-N strain is supplemented with exogenous *gacS in trans* (resulting in GS-N (pUCP18mpgacS)). Furthermore, the GS-SV (pUCP18mpgacS) strain produces thick biofilms, like its uncomplemented predecessor, but the degree of bumpiness on its surface is greatly diminished. Unfortunately, no simultaneous GS-SV control strain was cultured for this experiment, as such dramatic changes in biofilm morphology were not expected with complementation. The complemented strains produced a portion of their population as cells of filamentous morphology.

During the course of this study, a number of pegs were collected at 26 hours for each of the strains (4 for GS-SV (pUCP18mpgacS), 8 for all others) in order to test for statistically significant differences between the sizes of the biofilm population produced by each strain. Planktonic culture samples were also collected for all strains except GS-SV (pUCP18mpgacS). Since the complemented strains carried a plasmid, those cultures were grown in the presence of 500 ug/mL carbenicillin in order to exert a selective pressure for those cells carrying the pUCP18mpgacS construct. So as to minimize artefactual variation in the data through the presence of antibiotics in some cultures but not others, the GS-N strain was grown in the presence of 15 ug/mL gentamicin. PA14 could not be grown in the presence of any antibiotics as it carried no antibiotic resistance genes. The data resulting from this experiment can be viewed in Tables 15 and 16 in Appendix I. Upon examination of these data, one notes that the previously established relationships in terms of biofilm population remain unchanged amongst the complemented strains (i.e. GS-N (pUCP18mpgacS) and PA14 (pUCP18mpgacS) are not significantly different whereas GS-SV (pUCP18mpgacS) is significantly larger than both). However, the effect of antibiotics in the culture medium and/or the presence of the complementation construct can be seen to affect biofilm size (i.e. both PA14 (pUCP18mpgacS) and GS-N (pUCP18mpgacS) are significantly smaller in terms of biofilm population than is PA14). One also notes that there are no significant differences between the planktonic populations of any of the strains.

Figure 27. Low magnification environmental scanning electron micrographs of PA14, GS-N, PA14 (pUCP18mpgacS), GS-N (pUCP18mpgacS), and GS-SV (pUCP18mpgacS) pegs harvested at 26hr. Three pegs from each strain were studied. Each row represents a single strain. The columns are numbered 1,2, and 3 for the ready identification of individual images.

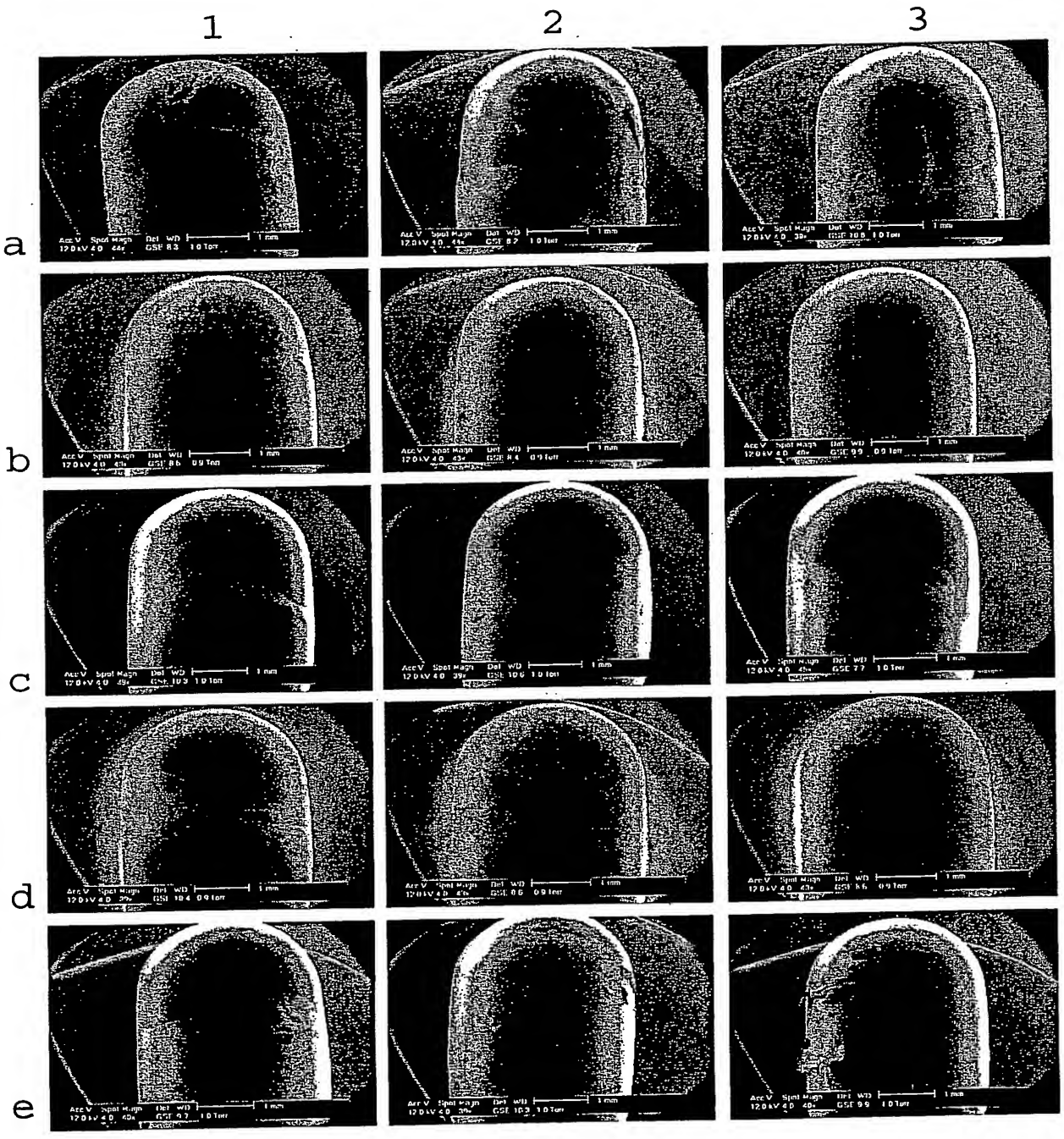
a.) PA14

b.) GS-N

c.) PA14 (pUCP18mpgacS)

d.) GS-N (pUCP18mpgacS). Note that complementation seems to partially rectify the coverage deficiencies of GS-N.

e.) GS-SV (pUCP18mpgacS). There is some surface bumpiness in the GS-SV biofilms, but far less than would normally be expected at 26 hrs.

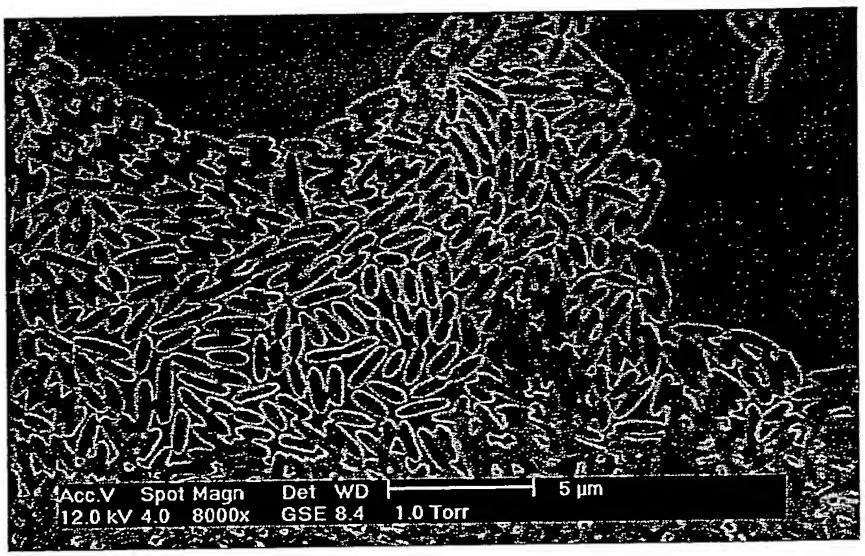


Attachment D

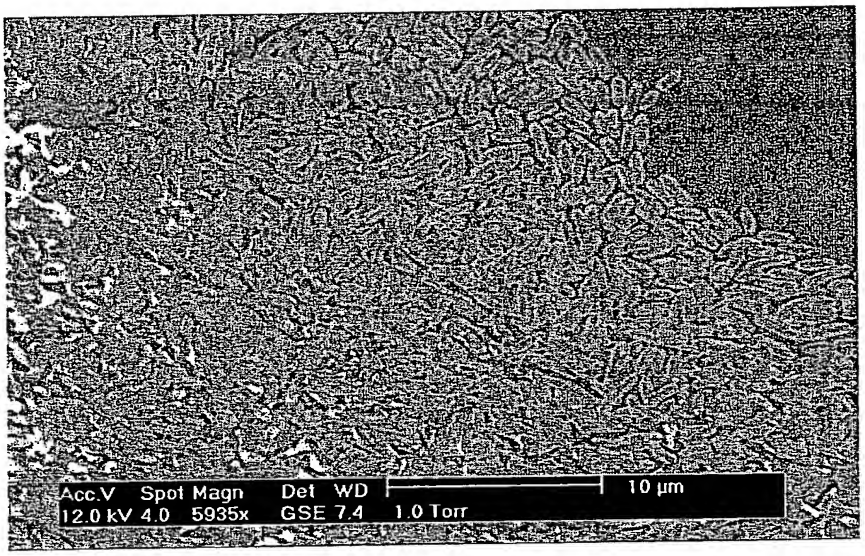
Figure 28. Environmental scanning electron micrographs of PA14 complemented with *gacS in trans* (PA14 (pUCP18mpgacS)) and grown in the presence of carbenicillin for 26 hours. PA14 (pUCP18mpgacS) produced biofilms that showed little difference from those of unaltered PA14 at 26 hours. There is some slight filamentation of cells.

a.) This image is purposely skewed in contrast so that the outline of individual cells is clear. Note how several of the cells are 2-3 times the length of the more normally proportioned cells.

b.) The edge of a PA14 (pUCP18mpgacS) biofilm at 26 hours incubation showing essentially normal structure and depth.


Acc.V Spot Magn Det WD 5 µm
12.0 kV 4.0 8000x GSE 8.4 1.0 Torr

a

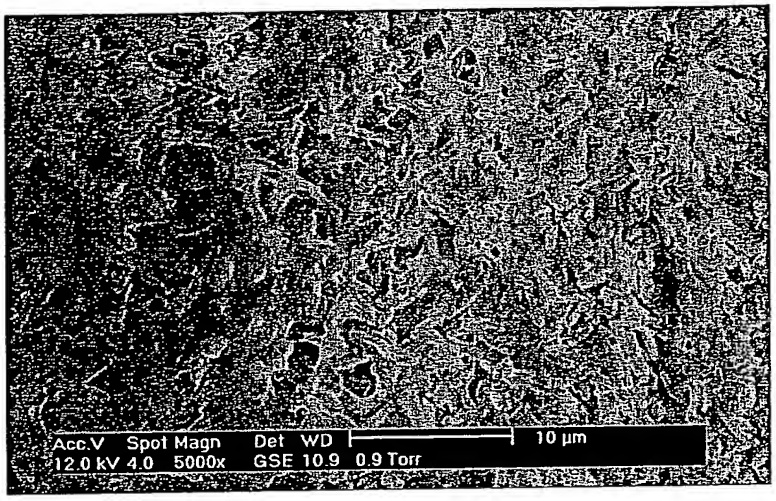

Acc.V Spot Magn Det WD 10 µm
12.0 kV 4.0 5935x GSE 7.4 1.0 Torr

b

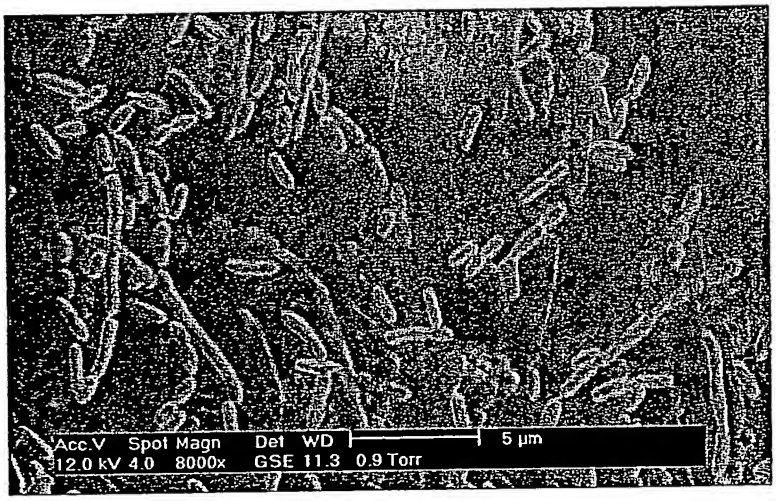
Figure 29. Environmental scanning electron micrographs of GS-N (pUCP18mpgacS) biofilms at 26 hrs. grown in the presence of carbenicillin.

a.) GS-N (pUCP18mpgacS) produced biofilms of much greater structure than did the uncomplemented GS-N at 26 hours but of less structure than PA14, PA14 (pUCP18mpgacS), or GS-SV (pUCP18mpgacS).

b.) Note the filamentation of cells is more severe in this strain than in PA14 (pUCP18mpgacS)


Acc.V Spot Magn Det WD 10 µm
12.0 kV 4.0 5000x GSE 10.9 0.9 Torr

a

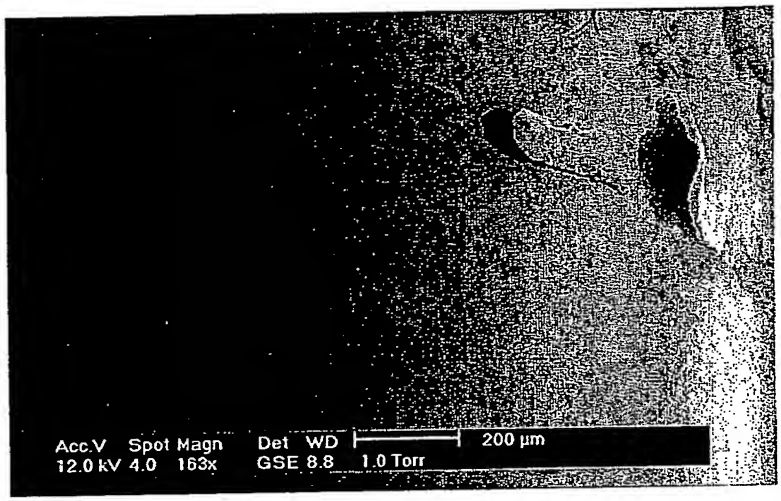

Acc.V Spot Magn Det WD 5 µm
12.0 kV 4.0 8000x GSE 11.3 0.9 Torr

b

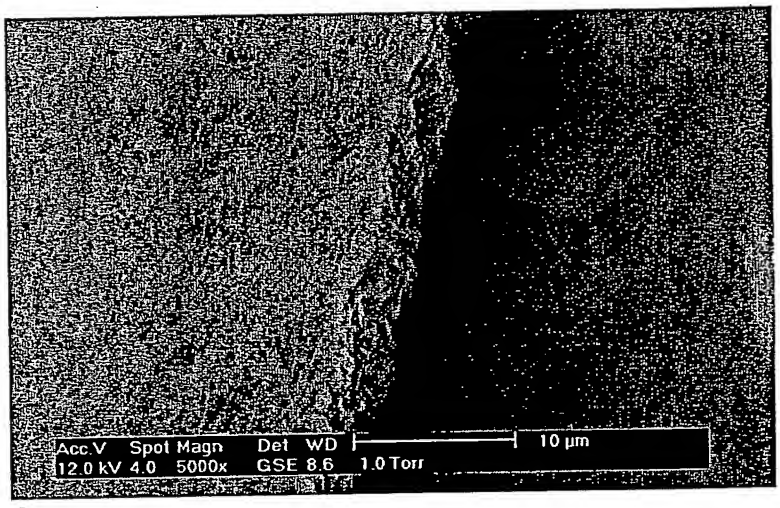
Figure 30. Environmental scanning electron micrographs of GS-SV (pUCP18mpgacS) biofilms grown for 26 hrs in the presence of carbenicillin.

a.) The biofilms formed by GS-SV (pUCP18mpgacS) are less bumpy than would be expected from the uncomplemented *gacS* mutant GS-SV at this timepoint.

b.) The films produced by GS-SV (pUCP18mpgacS) are very thick, as would be expected from previous experiments. The faint rectangular shape seen in this image was the result of "sample burn" by the electron beam when a high magnification image was being captured. This was much more of a problem when using ESEM imaging as opposed to standard SEM techniques.


Acc.V Spot Magn Det WD 200 µm
12.0 kV 4.0 163x GSE 8.8 1.0 Torr

a


Acc.V Spot Magn Det WD 10 µm
12.0 kV 4.0 5000x GSE 8.6 1.0 Torr

b

3.12 MIC and MBEC analysis

In addition to differences in growth and appearance of biofilms examined in previous sections, antibiotic sensitivity for PA14, GS-N and GS-SV was also measured. Minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) for a large number of antibiotics were determined as outlined in the Methods and Materials chapter. No consistent and significant differences in the MIC's or MBEC's were noted between PA14, GS-N, and GS-SV except as expected in the case of gentamicin resistance where the MIC and MBEC were observed to be significantly greater in the gacS mutant strains than in PA14 (Tables 6 and 7). In the first trial, it was noted that some of the biofilms (especially GS-SV) had sloughed off the pegs, leading to differences in innoculum size. To combat this, a second trial was conducted at an earlier time point (18 instead of 28 hrs.). Results of this time point (Table 7) generally showed lower biofilm but not planktonic resistance to antibiotics than those of the later culture, but again, no large or consistent differences between strains.

Table 6. Antibiotic sensitivity of P. aeruginosa strains PA14, GS-N, and GS-SV at 28 hours incubation in both the planktonic (MIC) and biofilm (MBEC) mode of growth

MIC (ug/mL)

ANTIBIOTIC	PA14	GS-N	GS-SV
amikacin	4	4	≤2
aztreonam	16	16	16
ceftazidime	4	16	4
ciprofloxacin	≤2	≤2	≤2
erythromycin	512	256	256
gentamicin	≤2	512	128
imipenem	≤2	≤2	≤2
piperacillin	16	16	8
polymixin B	≤2	≤2	4
tetracycline	64	64	64
tobramycin	≤2	≤2	≤2

MBEC (ug/mL)

ANTIBIOTIC	PA14	GS-N	GS-SV
amikacin	512	512	1024
aztreonam	>1024	>1024	>1024
ceftazidime	>1024	>1024	>1024
ciprofloxacin	32	64	32
erythromycin	>1024	>1024	>1024
gentamicin	128	>1024	>1024
imipenem	>1024	>1024	>1024
piperacillin	>1024	1024	>1024
polymixin B	128	256	512
tetracycline	>1024	>1024	1024
tobramycin	256	256	256

Table 7. Antibiotic sensitivity of *P. aeruginosa* strains PA14, GS-N, and GS-SV at 18 hours incubation in both the planktonic (MIC) and biofilm (MBEC) mode of growth

MIC (ug/mL)

ANTIBIOTIC	PA14	GS-N	GS-SV
amikacin	8	8	4
aztreonam	16	16	16
ceftazidime	4	4	4
ciprofloxacin	≤2	≤2	≤2
erythromycin	256	1024	256
gentamicin	≤2	1024	512
imipenem	≤2	≤2	≤2
piperacillin	16	256	8
polymixin B	≤2	≤2	≤2
tetracycline	64	64	64
tobramycin	≤2	≤2	≤2

MBEC (ug/mL)

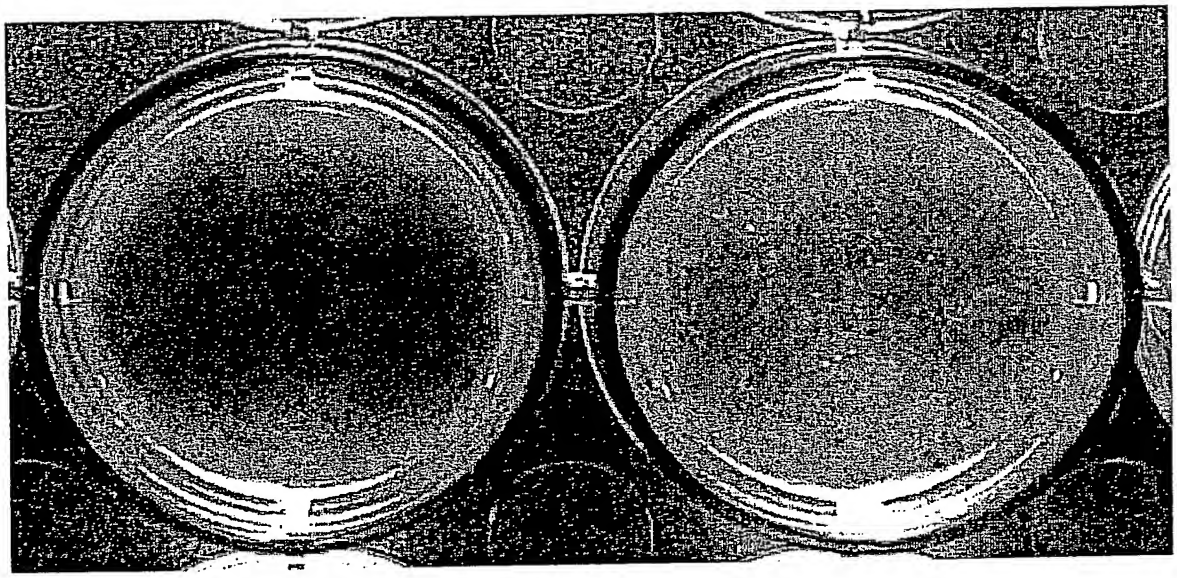
ANTIBIOTIC	PA14	GS-N	GS-SV
amikacin	32	32	64
aztreonam	>1024	>1024	1024
ceftazidime	>1024	>1024	>1024
ciprofloxacin	8	2	2
erythromycin	>1024	>1024	>1024
gentamicin	16	>1024	>1024
imipenem	>1024	>1024	>1024
piperacillin	>1024	>1024	>1024
polymixin B	16	16	32
tetracycline	1024	1024	1024
tobramycin	8	8	16

3.13 Colony morphology and broth culture characteristics

In accordance with the paper published by Drenkard and Ausbel (53) and the seeming similarity between the GS-SV phase variant in this study and the one they observed, a crude assay of aggregation in a non-agitated broth culture was undertaken (fig. 31). This culture was grown overnight at 37^{0}C. The broth culture was allowed to sit at room temperature without agitation for approximately 24 hours. Increased aggregation of GS-SV compared to GS-N was observed (Figure 31). When allowed to grow further at room temperature without agitation, the GS-SV well produced a pellicle over the top of the broth. The GS-N broth culture never formed any pellicle at all. It was also noted that both the GS-N and GS-SV strains had the ability to turn PIA and nutrient agar pink (Figure 33).

The complemented GS-SV strain (GS-SV (pUCP18mpgacS)) still flocculated, formed a pellicle, and sank in broth culture when that culture was left without agitation (Figures 31 and 32). This strain did, on occasion, revert back to its normal greenish hue (Figure 32). However, as shown in Figure (34), this complemented strain regained the ability to form "normal" colony morphologies when grown in broth culture for less than 48 hrs.

Figure 31. Aggregation assay of GS-SV and GS-N. Aggregates are observed in GS-SV (right) while none are seen in GS-N (left). No aggregates were noted in PA14 cultures (not shown)

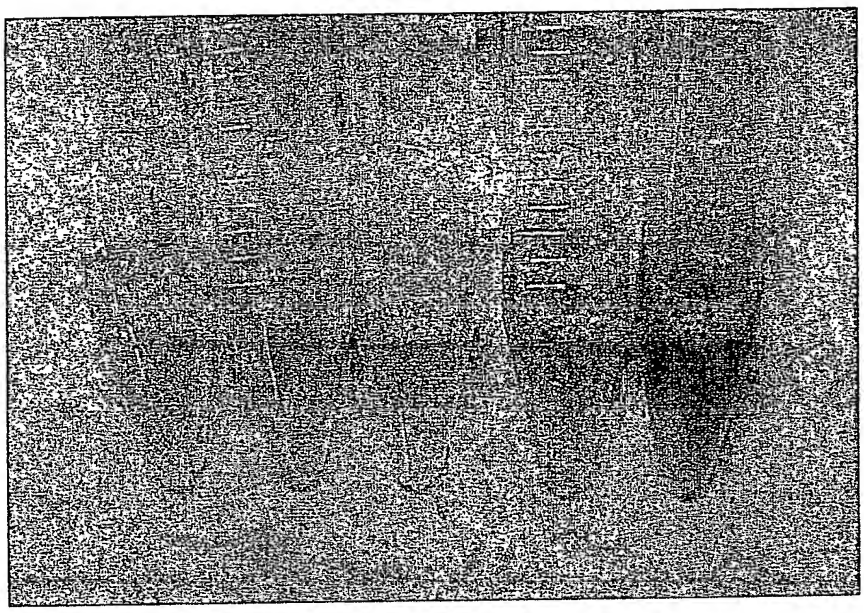


Attachment D

Figure 32. Broth culture characteristics of *P. aeruginosa* strains.

a.) Agitated 45 hr culture in nutrient broth plus appropriate antibiotics of the strains (from left to right) PA14, GS-N, PA14 (pUCP18mpgacS), GS-N (pUCP18mpgacS), GS-SV (pUCP18mpgacS). Note that the GS-SV (pUCP18mpgacS) culture is noticeably greener than the others, even PA14 and PA14 (pUCP18mpgacS).

b.) The same cultures in the same order as panel a., but they have been allowed to sit without agitation. Note the accumulation of material in the bottom of the tube containing GS-SV (pUCP18mpgacS) (far right). Also note the difference in colour is less noticeable than when the cultures were well suspended.



a

b

Figure 33. Colour change observed on nutrient agar. From bottom left and moving clockwise, the plates represent PA14, PA14 (pUCP18mpgacS), GS-N (pUCP18mpgacS) , and GS-N cultures grown on nutrient agar overnight at 37^{0}C. Note that the wildtype PA14 culture produces the most intense blue/green colour (presumably pyocyanin) while the mutant strains predominantly produce a red/brown pigment (presumably pyorubin). When introduced, the gacS complementation construct actually reduces the blue green colour of PA14 while having no discernible effect on the *gacS* mutant strain.

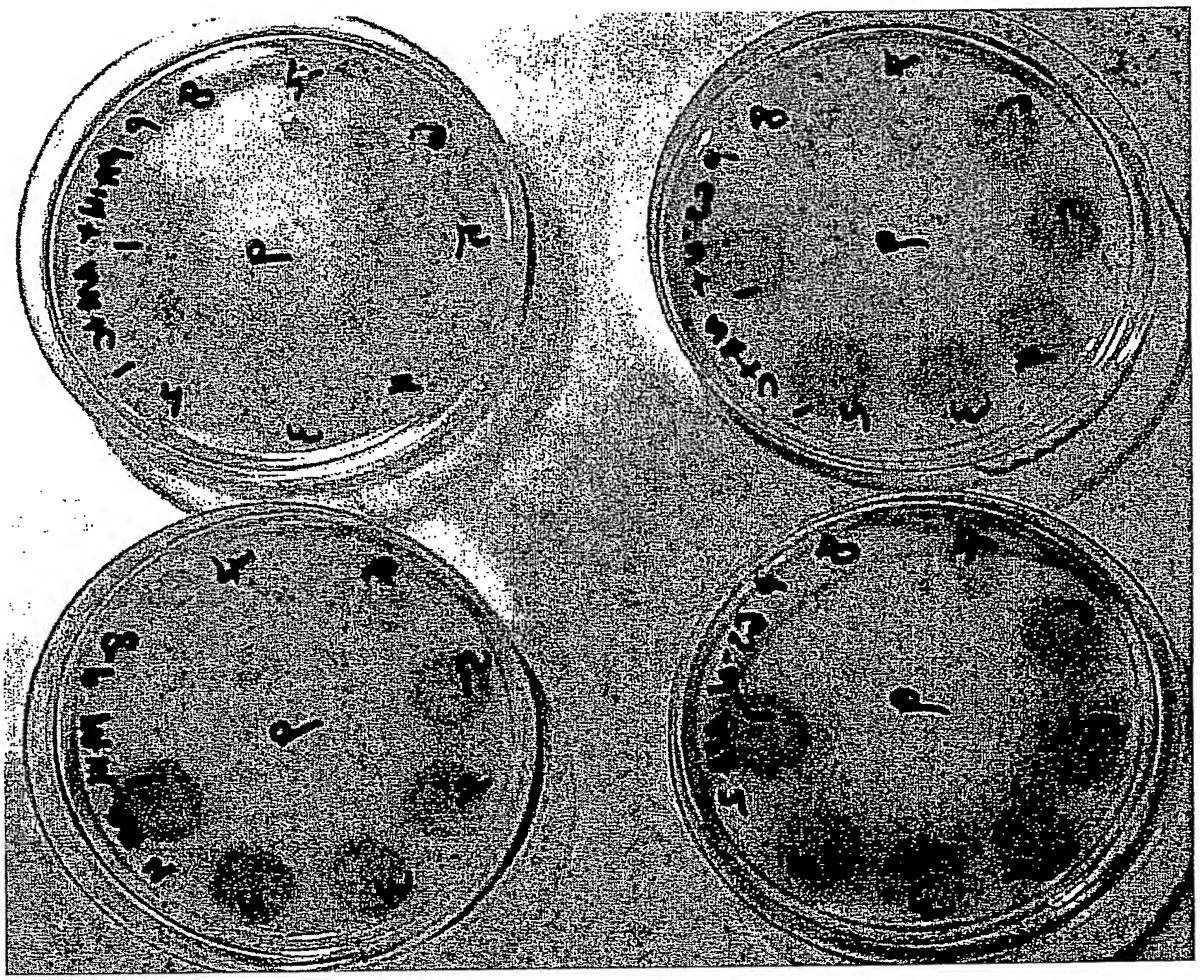
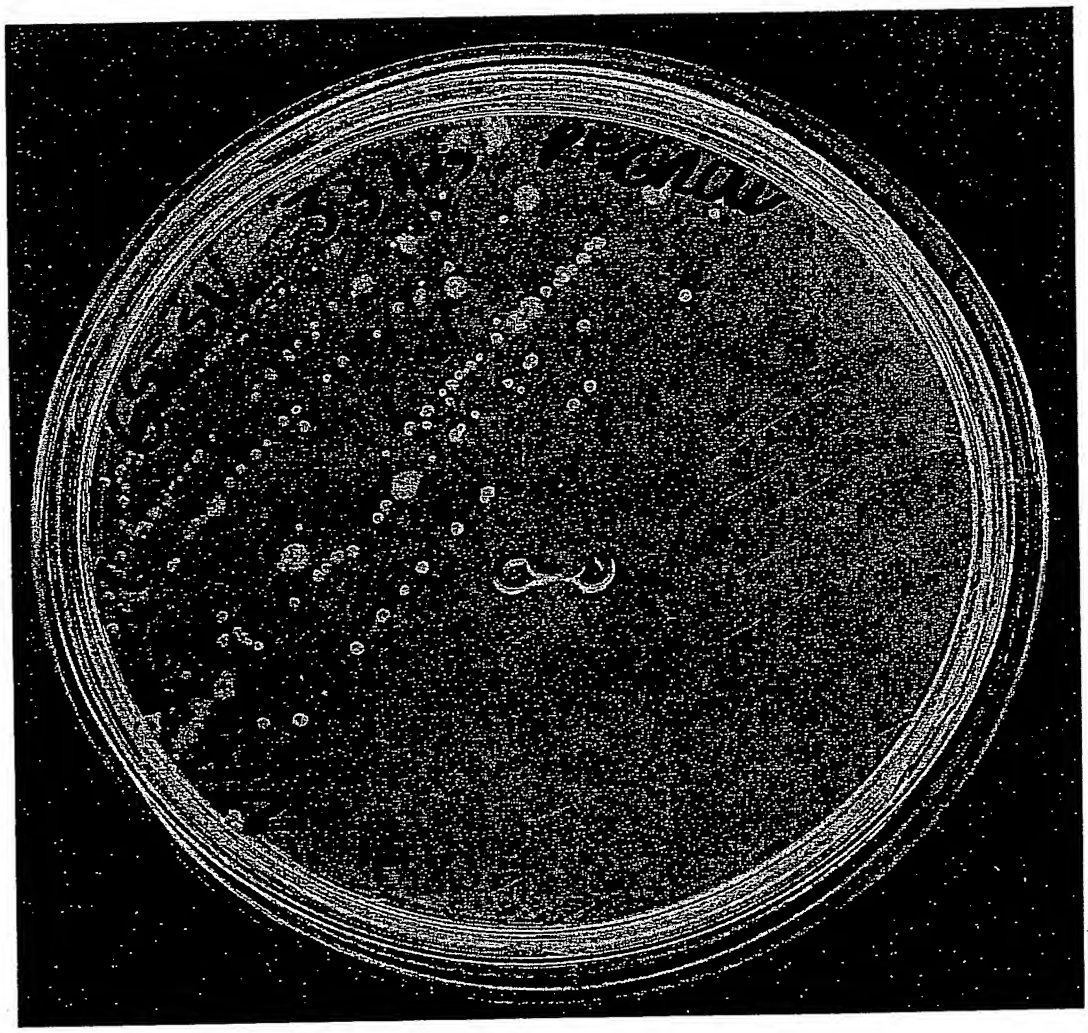


Figure 34. Nutrient agar plate culture of GS-SV (pUCP18mpgacS). A nutrient agar plate containing 15 ug/mL gentamicin and 500 ug/mL carbenicillin was streaked with broth culture from the GS-SV (pUCP18mpgacS) culture in Figure 32a. Notice that there are many colonies that show reversion back to the normal, larger morphology. This never occurred with the uncomplemented GS-SV strain, even when growth conditions were altered to promote reversion.



Attachment D

3.14 Exoprotease production

As *gacS* has been associated with secreted products (23,24,28), a crude assay of excreted protease was undertaken using milk agar plates. Bacterial cultures were spotted onto plates at dilutions from 10^1 to 10^4. Quantifiable and discrete zones of clearing consistently appeared at dilutions of 10^2 and 10^3. The diameter of the zone of clearing for all strains at this dilution was approximately 14 mm. No differences were noted between any of the strains in the size or quality of the zone of clearing.

4. DISCUSSION

4.1 In vivo analysis of the role of *gacA*⁻ in biofilm development

The potential for biofilm development in the context of an implant associated infection was initially explored using a *gacA* null mutant. The results of the initial rat implant experiment revealed that the *gacA*⁻ strain of PA14 produced smaller CFU/implant counts than did the wildtype strain. These findings, however were not confirmed by subsequent trials. It would seem that the viability of the *gacA*- strain was compromised in the initial experiment as it showed CFU/implant numbers similar to those of the negative control group (sterile saline). How this occurred is unknown. Alternatively, it is possible that spontaneous mutations in the Gac system were partially responsible for the results of the second and third trials, but this seems unlikely over the relatively short term of the experiment. It has been noted by other authors that spontaneous mutations occur in *gacS* and *gacA* under natural and laboratory conditions (54, 55, 56). It is possible that sufficient *gacS/A* mutations arose spontaneously in the wildtype PA14 strain to reduce any growth differences between that experimental group and the *gacA*- group. Still, this scenario is unlikely as *gacS/A* mutants seldom comprise the majority of a bacterial population (57), and these mutations are often associated with prolonged incubations and/or nutrient rich media (56). Neither of these conditions were satisfied in the rat's abdominal cavity. Moreover, this still does not explain why the *gacA*- strain in the initial experiment failed to persist in all but one replicate.

Scanning electron microscopy was used only to establish the presence of the biofilms on the wildtype implants. No images were taken of the *gacA*- implants. Given the relative scarcity of the biofilms and the degree to which host material accumulated on the implants,

it is unlikely that any such images could really provide insight into the structure of *gacA*- biofilms. In any case, the structure of *gacA*- biofilms has already been assessed using SEM by Parkins (30). No instances of phase variation were seen, although no particular effort was made to observe this phenomenon in these experiments.

4.2 Analysis of *gacS*- strains of *P. aeruginosa*

To generate the mutant strain, a gentamicin resistance cassette was inserted into the *gacS* gene, 521 bp from its transcriptional start point. This region is well within the protein coding section of the gene. With PCR, the quantity of amplicon produced from various mutant clones seemed to vary with the position of the reaction tube in the thermocycler's heating block. This variability was not noted in the amplification of the wildtype gene. Two possible explanations for this variability exist. One, the extra length of the template coupled with its relative G-C rich sequence resulted in a less efficient amplification. Two, the incorporation of gm^R introduced structural issues into the amplicon, rendering it difficult to amplify. The latter explanation would seem more likely as multiple attempts were made to sequence the mutated gene, before success was Obtained. Sequencing the amplicon of wildtype template did not produce these difficulties.

The *gacS*- strains generated in this study produced a light yellow colour when grown in LB or TSB liquid culture. This contrasts with the green colour of the wildtype *P. aeruginosa* PA14 grown under the same conditions. This is not surprising as the Gac system has been associated with pigment production (66). Complementation with functional *gacS* did restore some green colour to the cultures, but this was inconsistent and variable in intensity.

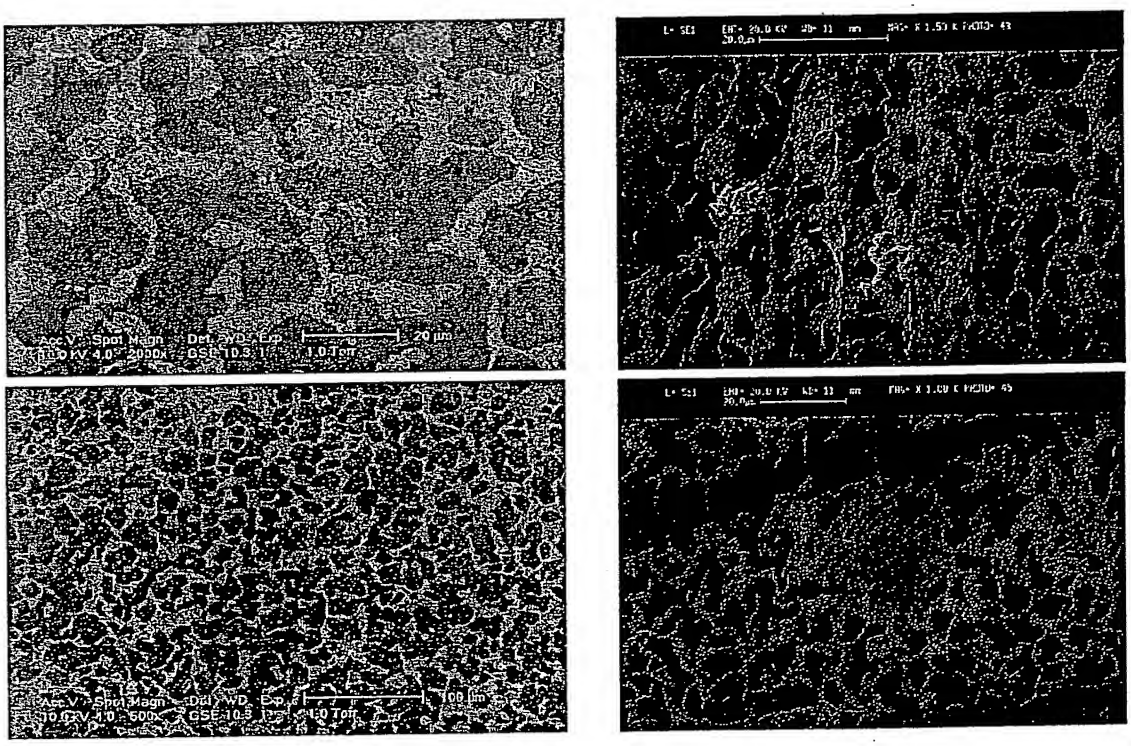
The results of the PA14 vs. *gacS*- vs. *gacA*- growth curve do not indicate any significant differences between the strains. This differs from observations made by Parkins (30) although differences in methodology may, in part, explain the discrepancies between the two studies. Parkins (30) used a vigorous rinse step that was not employed in this study. This study used a rinse step consisting of dipping the peg/implant in a pool of saline rather than energetically swirling the peg within the pool. This change was justified through the observation that the films detached easily from the polystyrene peg. Vigorous rinsing would lead to underestimation of biofilm size, as a portion of the film present on the peg would be left in the rinse solution. Furthermore, it is evident that the presence of antibiotics in the liquid media used in the CBD can cause an artefactual reduction in biofilm size without concurrent reduction in planktonic populations (see Appendix I Tables 15 and 16). While the nature of this phenomenon is beyond the scope of this study, it may explain why the results of this study differ from previous work. The ready isolation of gentamicin resistant bacteria and the presence of distinct phenotypes even after prolonged incubation in the absence of gentamicin argues that this decision was not reckless or frivolous. In any case, the curves likely still underestimate CFU/peg, as mutations in the Gac system have been associated with the generation of viable but nonculturable (VBNC) cells (58, Marques unpublished). These cells are active metabolically, but are incapable of reproducing. VBNC cells were left uncounted by the colony based methods employed by this study. Although films detached easily from their peg, they did not dissociate into individual cells readily. This was particularly true of older PA14 films (e.g. >24 hrs. old) and those generated by the GS-SV phase variant. Despite vigorous vortexing and 30 minutes of sonication, large fragments of biofilm were grossly visible in the resuspension

solution. The cells within these fragments could never be properly resuspended and, therefore, never enumerated.

While the GS-SV phase variant showed approximately a one $\log_{10}$ unit increase in biofilm CFU/peg over GS-N and PA14, its planktonic CFU/mL counts were usually either at or below the levels of the other two strains. The simplest explanation for this is that the GS-SV cells, with their natural tendency to aggregate and sink, were less likely to be enumerated by the dilution based assay used in this study. Cells that dropped to the floor of the CBD or dilution wells would likely not be transferred to the agar plates used to determine planktonic numbers.

In the late stages of growth, GS-N biofilms dropped in CFU/peg relative to PA14 (see Figure 13). The growth curves, however, show a much smaller difference in biofilm size than was indicated by the scanning electron micrographs. In any case, the differences between GS-N and PA14 demonstrated in Figure 13 and Table 14 were not consistent. Figure 12 indicates GS-N biofilms (labelled *gacS*⁻ in that Figure) are almost identical in size to PA14 films, as did many informal replicates of the growth curve experiments. Yet, scanning electron micrographs consistently showed that GS-N films were slower to develop. What is consistent is the structure of GS-N films when considered across two species of pseudomonads. Figure 35 shows scanning electron micrographs of the biofilms generated by the GS-N strain developed in this study. The same Figure also displays biofilms generated by the *P. chlororaphis* O6 *gacS*- mutant developed by Dr. Anne Anderson. These micrographs were kindly donated with permission by Dr. L. Marques. Both mutant strains generate a thin reticulated pattern over the surface of the peg with occasional "chunks".

Figure 35. Comparable biofilms structures formed by two *gacS* mutants at 24 hrs. The left column shows GS-N biofilms. The right column shows *P. chlororaphis* O6 *gacS* biofilms. Note the similarity in structure.



4.3 The GS-SV phase variant

Phase variation is a strategy that bacteria use to survive changing environmental conditions. This process consists of the creation of a phenotypically diverse population of a single species of organism (70). Phase variation is known to be increased in biofilm populations relative to planktonic populations (59). Certainly this seemed to be the case in this study, where almost all the phase variant colonies were observed in biofilm preparations. An inactivating mutation of *pheN* (a homologue of *gacS* (24)) in *P. tolassii* has been shown to lead to phase variation (55). Sanchez-Contreras found a *gacA*$^-$ phase variant that flocculated and sank in static broth culture (54), similar to the GS-SV variant found in this study and a *gacS*$^-$ phase variant generated from *P. chlororaphis* (Marques unpublished). The GS-SV phase variant from this study would also seem to bear a resemblance to those found by Deziel in *P. aeruginosa* 57RP (59) and Drenkard in cystic fibrosis isolates (53). The GS-SV variant showed colony morphology, agglutination and biofilm formation ability similar to that of Drenkard and Deziel with the pellicle forming characteristics of Deziel's variant. Unlike most other phase variants, GS-SV did not revert to normal morphology, even after 45 days serial plating at room temperature. This far exceeds the 5 day standard used by Drenkard. Interestingly, the phase variants from this study were seen almost entirely in the *gacS*$^-$ strain. Given that the GS-SV phase variant was effectively generated only out of the GS-N strain, that the phase variants tended to be seen in the late stages of growth, and that these variants failed to revert after 45 days, it is reasonable to speculate that the *gacS* gene is involved in the reversion of phase variants back to their "normal" form. This theory is similar to the one proposed by Drenkard for the *pvrR* gene. The presence of revertant colonies in the GS-SV (pUCP18mpgacS) strain, after

less than 48hrs. incubation in broth culture, would seem to verify the plausibility of this hypothesis.

There has been work to suggest that *rpoS* has been involved with adaptive mutation (60). There has also been work linking *rpoS* with the *gacS* gene (25, 61). An *rpoS* mutant generated by Heydorn produces very thick biofilms (if not bumpy) as does the GS-SV phase variant (78). The work done by Rashid and Kornberg links *ppk* to *rpoS* (62)which, in turn, may be linked to the function of *gacS*. A phase variant has also been seen in a *ppk* knockout by Rashid and Kornberg that resembles Deziel's variant. The *gacS* phase variant (GS-SV) generated in this study is similar in some respects to Deziel's. The relationship between *ppk*, *rpoS*, and *gacS* is still undefined. However, when multiple studies are considered, there is significant circumstantial evidence to indicate a link between these 3 genes.

With the exception of late stage GS-N cultures, the scanning electron micrographs taken of CBD pegs would seem to indicate that the numbers of bacteria present on the surface of the pegs are disproportionately large compared to the CFU/peg counts determined by the biofilm growth curves. This could be the result of a large population of VBNC cells. The relationship between the Gac system and the accumulation of these cells has been noted by others (58, Marques unpublished). Inadequate biofilm dispersion would also contribute to this problem for both the PA14 and *gacS* cultures. When viewing these images, one also notes the speed with which large biofilms are formed by the GS-SV phase variant. Though no evidence of increased GS-N or GS-SV planktonic growth was seen in this study, Gac system mutants have been shown by other investigators to have an advantage over wildtype at early stages in their growth curve (57). Furthermore, increased biofilm mass has been

seen in *P. aeruginosa* phase variants similar to those collected out of cystic fibrosis patients (53). In this regard, the phase variants found by Drenkard would seem to be similar to the GS-SV variant generated by this study.

In the later stages of biofilm development, other changes are apparent. Compared to PA14, GS-N cultures never produced a well organized biofilm. GS-N cultures at 24 hrs incubation and viewed under ESEM produced pegs that showed little to none of the peg surface covered by an organized biofilm. In fact, most of the surface of the peg was covered with a thin, reticulated monolayer of cells (Figure 35), if not just a scattering of individual cells. Initially, it was thought that this was the result of a large primary film sloughing off and being replaced by a younger secondary film. It was not expected to be a widespread phenomenon. However, when more pegs were collected at a similar time point and viewed under the same conditions, all showed a similar structure with no obvious film fragments in the broth at the time of collection. Complementation experiments showed that while this phenotype did not completely return to that of the wildtype organism, coverage was improved at the 26 hour time point (Figure 27). The GS-SV variant produces a very different film in the late stages of development. It produces massive films with a bumpy surface. One could theorize that this appearance and the tendency for these films to slough could be related. The Gac system has been shown to be involved in alginate synthesis (29, 72), although there is controversy over this point (41). Alginate is a large component of the extracellular matrix material in biofilms (63). The Gac system is also well established as a regulator for other extracellular products (66). With this in mind, it is reasonable to speculate that a *gacS* mutant, would produce altered extracellular polymeric substances (EPS). This could account for the increased sloughing of GS-SV films, and

possibly the underdevelopment of late stage GS-N films. Furthermore, given that these films were induced on the pegs by a shear force that reversed direction every 3-4 seconds, it is possible that the bumps on the surface of GS-SV biofilms were the result of fluid dynamics around the peg. Turbulent flow may have been created around the peg, promoting erosion of the biofilm into mounds and pits. The relatively uniform nature of the high and low points on the late-stage GS-SV biofilms supports this theory. The complemented strain of GS-SV (GS-SV (pUCP18mpgacS)) produced a thick biofilm, but it did not produce such severe topography as did the unaltered GS-SV. Figure 34 would suggest that some, but not the majority of the cells involved in this biofilm, would have reverted back to their "non-phase variant" phenotype. So, it would seem that the lack of bumpiness on the GS-SV (pUCP18mpgacS) biofilms is most likely the direct result of the presence of a normal *gacS* gene.

4.4 Role of antibiotics in the culture medium

As an alternative explanation for differences observed in biofilm appearance, one could argue that the presence of antibiotics in the culture media altered the structure of the GS-SV (pUCP18mpgacS), and possibly the GS-N (pUCP18mpgacS) biofilms. Certainly, there is evidence to indicate that the presence of antibiotics inhibits the formation of biofilms under the conditions used in this study (see Appendix I). If one assumes that the bumpiness of GS-SV biofilms is depended on cell numbers, then one could make a reasonable argument for the lack of bumpiness in the GS-SV (pUCP18mpgacS) biofilms being an artefact. Still, this seems unlikely. While the CFU/peg of GS-SV (pUCP18mpgacS) biofilms grown in the presence of carbenicillin is somewhat smaller than

that of GS-SV biofilms grown in the absence of any antibiotic, the GS-SV (pUCP18mpgacS) films are still thick enough to show dessication cracking. Empirically, this event would seem to coincide with the appearance of a bumpy surface on the biofilm. In any case, the answer to this question could be easily determined by conducting an experiment where GS-SV biofilms are grown in the presence of gentamicin and GS-SV (pUCP18mpgacS) biofilms are grown in the presence of carbenicillin. One would then examine pegs from each culture and assess the relative "bumpiness" of each. So long as the CFU/peg of each culture is similar and pegs are collected at the same time point, the results should be valid.

Upon observation of the scanning electron micrographs of the complemented strains, the presence of cells with an abnormal filamentous morphology was observed (Figures 28 and 29). This is most likely the result of sub-lethal concentrations of carbenicillin (a β-lactam antibiotic) in the growth media. A very similar morphology has been seen by Horii in *Pseudomonas aeruginosa* exposed to ceftazidime (another β-lactam antibiotic) (80). One notes that the filamentous cell morphologies are most noticeable in the GS-N (pUCP18mpgacS) and GS-SV (pUCP18mpgacS) films while the same morphology is quite rare in the PA14 (pUCP18mpgacS) films. It is possible that through a technical error on the part of the experimenter, the concentration of carbenicillin in the PA14 (pUCP18mpgacS) culture was less than that in the other cultures. Alternatively, one could link the filamentation to the relative load of pUCP18mpgacS present in each strain, as the PA14 (pUCP18mpgacS) strain seemed to carry less of this construct than did the other two strains. Still, how vector load could cause this level of change in cellular morphology is

unknown. It is unknown what effect cell filamentation has on biofilm formation by *P. aeruginosa*.

Scanning electron microscopy did induce dessication artefact in the biofilms as can be seen by the cracking and peeling of the films and the indentation of the cells themselves (puffed wheat appearance). This makes the native structure of the films difficult to assess. Confocal scanning laser microscopy (CSLM) would be a better assay of the structure of the normal, hydrated film.

It has already been established the *P. aeruginosa* biofilms are more resistant to antibiotics than are the planktonic cells (30). One group (42) has suggested that a *gacS* mutant of *P. aeruginosa* is more susceptible to gentamicin and amikacin. While gentamicin sensitivity could not be assessed due to the presence of a gentamicin resistance cassette in the mutant strains, a two-fold reduction in amikacin MIC was observed in GS-SV, consistent with one of Brinkman's (42) observations for that variant. In addition, an unpublished observation made in Dr. Doug Storey's laboratory at the University of Calgary suggests that the swarming behaviour of the GS-SV variant is similar to that described by Brinkman. Interestingly, the MBEC for amikacin in GS-SV showed a twofold increase, the opposite of what one would expect. Never the less, these are minor changes. Anything less than a four-fold change was considered insignificant for the purposes of this study. According to these standards, it is clear that *gacS* mutation does not substantially alter antibiotic sensitivity. Younger biofilms were more sensitive to antibiotics than were older ones. This variation of biofilm antibiotic sensitivity with age has been observed previously (81). Phase variation, other than that responsible for the generation of the GS-SV variant, could account for this. Certainly Drenkard (53) found that phase variation could affect *P.*

aeruginosa antibiotic sensitivity. Furthermore, Dr. L. Marques has found that subjecting *gacS* mutants of *P. chlororaphis* O6 to certain biocides can induce phase variation (unpublished). Walters has postulated that oxygen limitation and low metabolic rate in the deep regions of the biofilm contribute to it's resistance to antibiotics (71). If this is the case, then the greater antibiotic resistance of older compared to younger films may indicate that the oxygen concentration and metabolic activity gradients take time to become established in a biofilm.

Parkins (30) and Walters (71) have shown that biofilm resistance to antibiotics is not simply a matter of a diffusional barrier. The data from this study support this argument. GS-SV biofilms, which were obviously thicker than PA14 or GS-N biofilms of the same age, were not significantly more or less susceptible to antibiotics compared to the other strains.

Both mutants showed the ability to turn PIA pink in the area surrounding the colonies. The wildtype PA14 did not do this. In consultation with Mr. John Ceballos of Becton Dickinson Diagnostic Systems, it is speculated that the pink colour may be the result of the production of pyrubin, a red diffusible pigment. This pigment has been seen when relevant microorganisms are grown on Bacto Pseudomonas Agar P (P agar) (72). P agar is a pseudomonas specific medium that enhances the production of pyocyanin, like PIA. In fact, the only difference between P agar and PIA is the inclusion of irgasan, an antimicrobial used to inhibit the growth of non-pseudomonas organisms.

4.5 The Gac system and exoprotease production

The Gac system has been associated with secreted proteases (22, 64, 25, 28, 65, 66) in the past. Because it could be done rapidly, cheaply, and easily, a crude assay of the quantity of secreted proteases was conducted. The procedure for this was modified from the methods of Corbell (22). No significant differences were seen between the mutant and wildtype strains. Still this was a crude assay, and more sensitive methods could be employed. This may also reflect species differences, as the work linking the Gac system with exoprotease production was done in Pseudomonas species other than aeruginosa (66).

4.6 Future work

Gac mutations occur spontaneously in nature (56, 54, 57) and those mutations seem to promote the survival the population as a whole (57). Phase variation is common in gram-negative bacterial populations, and is often the result of genomic rearrangements (67). This study and others (55) have provided evidence that supports the hypothesis that the Gac system, and potentially the mutation of that system, is involved in phase variation. The interplay of Gac mutations and phase variation as it pertains to the survival of bacterial populations under certain environmental circumstances is not well understood. Further investigation into this area may lead to discoveries in the areas of chronic infection, industrial fermentation, biofouling, and plant-microbe interactions. This study has shown that phase variation can have an enormous effect on the structure of a biofilm. It would seem that further investigation of the function of the Gac system in the context of the biofilm mode of growth will be done hand-in-hand with the study of phase variation in biofilms.

Although outside the scope of this study, the mechanics of the Gac system are still largely unknown. Certainly the Gac system has been linked with *rpoS* (25, 61). The gene *rpoS* has, in turn, been linked with *ppk*. Currently, there is work being done by Dr. L. Marques that may link *gacS* to *ppx*, a regulator of *ppk*. Furthermore, mutations in the Gac system can be suppressed by other factors (68). Even the signal sensed by GacS remains unknown, although work by Koch suggests that such a signal molecule may be found in sugar beet seed exudate (69). Understanding the mechanics of the Gac system should allow further insight into its role in biofilm formation and phase variation.

The mechanism by which the growth of the GS-N film is inhibited or the film itself degrades should be investigated further. It may be that the film simply detaches en masse from the peg. If this is the case, then the sloughing and bumpiness of GS-SV biofilms and the degradation of GS-N biofilms may both be the result of the *gacS* mutation. Still, no sloughed films were observed in the broth culture component of CBD containing GS-N cultures. Perhaps one can best accommodate all observations with the theory that GS-N cultures do not easily form structured biofilms because the mutant cells are sloughed or torn from the substrate soon after the process of biofilm formation is initiated. The GS-SV phase variant overcomes this deficiency through as yet undefined traits controlled in part by the phase variation process, thus producing massive biofilms that are still sensitive to mechanical removal.

The sloughing of the GS-SV variants, or cells like them, may be important to the ability of biofilms to "seed" new areas. Small numbers of cells that have a tendency to slough from the original film, aggregate together while in suspension, and adhere to a new surface, would promote biofilm expansion. Further studies into the circumstances of spontaneous

Gac mutation and/or phase variation may elucidate the role of these phenomenae in natural biofilms.

Whatever further work is conducted, it may be useful to refine or re-design the pUCP18mpgacS construct. The results of the complementation experiments using the original construct are inconsistent at best. For example, the complemented GS-SV strain can be, at late stages of broth culture growth, a greener hue than is the complemented GS-N (pUCP18mpgacS) and other GS-SV cultures grown under the same conditions. This occurs despite the fact that both strains were conjugated with the same *E. coli* SM10 clone. Furthermore, the choice of pUCP18 as a complementation vector was, perhaps, unfortunate. Most of the problems with this vector stem from the use of β-lactamase as a selectable marker (31). The mutant strains constructed in the course of this study are selected by means of gentamicin resistance. If one means to select for a gentamicin resistant *P. aeruginosa* mutant that carries a complementation construct coding for a β-lactamase, then one must select with a combination of gentamicin and carbenicillin (or other appropriate β-lactam antibiotic). This combination of aminoglycoside and β-lactam is known to be synergistic in its antimicrobial activity (73). As such, the combination may retard growth of complemented mutants to an unacceptable level – especially if one is doing experiments dependent upon growth rate. *Pseudomonas aeruginosa* has a high natural tolerance for β-lactams (31). Any condition that increases *P. aeruginosa*'s inherent tolerance for these drugs (e.g. biofilm mode of growth, accumulation of extracellular β-lactamases from transformants in old broth cultures) could lead to significant numbers of cells that do not carry the construct. Moreover, these conditions could allow transformed strains to largely "dump" the vector. Perhaps future studies could consider the use of other

vectors (e.g. pUCP28T or pUCP29T) that have selectable markers other than a β-lactamase.

Finally, work needs to be done on the methodologies involved in biofilm studies. Although convenient, the CBD produces films that vary in their peg coverage depending on the position of the peg within the device. This can be partially overcome by ensuring that the researcher selects pegs from the same location on each device when doing comparative experiments. While counting CFU/peg is convenient, it may be inadequate in some cases. Non-dissociating film fragments and VBNC cells lead to problems with underestimation of biofilm size. The current alternative is to do manual counts using viable/non-viable staining. This technique is very labour intensive. Some new alternative will have to be found. Scanning electron microscopy is very useful in studying biofilm structure. Still, it introduces artefact through dessication and does not allow for a cross-sectional view of the film. The use of confocal scanning laser microscopy instead of scanning electron microscopy would allow more insight into the structure of biofilms.

Despite the limitations in methodology that have come to light at the end of this study, there is no doubt that the approach taken has shown an important role for *gacS* in biofilm development in *P. aeruginosa*. Clearly, *gacS* inactivation led to the accumulation of a phenotypically stable, small colony phase variant that produced biofilms that developed remarkably more quickly than in unaltered *P. aeruginosa* PA14 and that developed a much greater biomass. The exact mechanism by which this occurs still needs to be elucidated but an important first step has been achieved.

5. References

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6. Appendix I: statistical analyses

Rat implant experiments

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
gacA- vs PA14	-3.92	10.2	P < 0.001
gacA- vs control	-0.0109	0.0285	P > 0.05
control vs PA14	-3.90	10.2	P < 0.001

Table 8. Significance of difference on the original rat implant experiment expressed as CFU/implant in $\log_{10}$. Note that the control and *gacA*⁻ groups are similar in magnitude.

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
cntrl vs gacA-	-2.32	2.97	P > 0.05
cntrl vs PA14	-2.31	---	P > 0.05
PA14 vs gacA-	-0.0114	---	P > 0.05

Table 9. Significance of difference on the first confirmatory trial ($\log_{10}$). Note none of the groups are significantly different from each other.

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
cntrl vs PA14	-4.10	5.89	P < 0.01
cntrl vs gacA-	-2.97	4.27	P < 0.05
gacA- vs PA14	-1.13	1.54	P > 0.05

Table 10. Significance of difference on the second confirmatory trial ($\log_{10}$). There is not significant difference between the *gacA*⁻ and PA14 groups

Planktonic and biofilm growth of PA14 vs GS-N vs GS-SV

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
PA14 vs GS-N	-0.4051	5.254	P < 0.01
PA14 vs GS-SV	-0.1713	2.222	P > 0.05
GS-SV vs GS-N	-0.2337	3.032	P < 0.05

Table 11. Planktonic growth - CFU/mL at 11 hrs. (log_{10}). GS-SV and PA14 are slightly lower than GS-N

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
GS-N vs GS-SV	-1.089	7.330	P < 0.001
GS-N vs PA14	-0.3881	2.611	P > 0.05
PA14 vs GS-SV	-0.7014	4.719	P < 0.01

Table 12. Biofilm growth - CFU/peg at 11 hrs. (log_{10}). GS-N and PA14 are lower than GS-SV.

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
GS-SV vs GS-N	-0.6172	7.332	P < 0.001
GS-SV vs PA14	-0.1812	2.153	P > 0.05
PA14 vs GS-N	-0.4360	5.179	P < 0.001

Table 13. Planktonic growth – CFU/mL at 20 hrs. (log_{10}). Like the 11 hr. point, GS-SV and PA14 are lower than GS-N.

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
GS-N vs GS-SV	-1.465	17.16	$P < 0.001$
GS-N vs PA14	-0.2790	3.268	$P < 0.05$
PA14 vs GS-SV	-1.186	13.89	$P < 0.001$

Table 14. Biofilm growth - CFU/peg at 20 hrs. (log_{10}). GS-SV is more than 1 log_{10} larger than PA14 or GS-N.

Planktonic and biofilm growth of standard and complemented strains

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
PA14 (pUCP18mpgacS) vs GS-N	-0.243	1.95	P > 0.05
PA14 (pUCP18mpgacS) vs GS-N (pUCP18mpgacS)	-0.229	---	P > 0.05
PA14 (pUCP18mpgacS) vs PA14	-0.0242	---	P > 0.05
PA14 vs GS-N	-0.219	---	P > 0.05
PA14 vs GS-N (pUCP18mpgacS)	-0.205	---	P > 0.05
GS-N (pUCP18mpgacS) vs GS-N	-0.0139	---	P > 0.05

Table 15. Twenty six hour planktonic counts in CFU/mL (log_{10}) for complemented strains. Note that there are no significant differences between strains in this parameter. All cultures except PA14 contained either 15 ug/mL gentamicin (GS-N) or 500 ug/mL carbenicillin (all others). There was no planktonic data for GS-SV or GS-SV (pUCP18mpgacS).

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
GS-N vs GS-SV (pUCP18mpgacS)	-1.12	9.61	P < 0.001
GS-N vs PA14	-1.00	10.5	P < 0.001
GS-N vs PA14 (pUCP18mpgacS)	-0.326	3.43	P > 0.05
GS-N vs GS-N (pUCP18mpgacS)	-0.116	---	P > 0.05
GS-N (pUCP18mpgacS) vs GS-SV (pUCP18mpgacS)	-1.00	8.62	P < 0.001
GS-N (pUCP18mpgacS) vs PA14	-0.885	9.31	P < 0.001
GS-N (pUCP18mpgacS) vs PA14 (pUCP18mpgacS)	-0.210	---	P > 0.05
PA14 (pUCP18mpgacS) vs GS-SV (pUCP18mpgacS)	-0.793	6.81	P < 0.001
PA14 (pUCP18mpgacS) vs PA14	-0.675	7.10	P < 0.001
PA14 vs GS-SV (pUCP18mpgacS)	-0.119	1.02	P > 0.05

Table 16. Twenty six hour biofilm counts in CFU/peg (log_{10}) for complemented strains. Strains grown in antibiotics (all except PA14) show lower CFU/peg than those grown in the absence of antibiotics. This is especially noticeable in PA14 vs. PA14 (pUCP18mpgacS). Furthermore, GS-N grown in gentamicin is significantly lower than PA14 (no antibiotics in media) but not significantly lower than PA14 (pUCP18mpgacS) (carbenicillin in media). This argues against a reduction in biofilm mass as a result of the presence of the pUCP18mpgacS vector. However, there was no significant difference between GS-N and GS-N (pUCP18mpgacS), which contradicts the ESEM data. GS-SV was significantly greater than all strains except PA14 which was grown in the absence of antibiotics.